Isolation of Ovine Hemoglobin, its Apoglobins and Peptides, for the Determination of Antimicrobial Activities

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Abstract

The objective of the research presented here was to investigate the properties of ovine hemoglobin, its subunits and its peptides as potential antimicrobial therapeutics or biopreservatives. This objective addresses two issues. The first is the growing lack of novel and effective antimicrobials against drug resistant microorganisms (superbugs); ovine hemoglobin and its components may provide an effective alternative. The second is the large volume of ovine blood generated from sheep slaughter in New Zealand, from which, currently only low value products such as blood meal are made; it is proposed that this blood be used as a source of antimicrobial peptides - high value products.

The research was divided into three parts. First, ovine hemoglobin was isolated from whole blood using isotonic ammonium chloride lysis of erythrocytes and the subunits were separated and de-hemed by acid acetone precipitation. Two conditions for pepsin digestion of hemoglobin into short random coiled peptides were also identified - hemoglobin as a starting substrate in its native and denatured conformations.

Secondly, the alpha and beta apoglobins were separated into their respective fractions by semi-preparative RP-HPLC. The kinetics of the two pepsin digestion conditions were also compared by RP-HPLC and it was found that denatured hemoglobin is digested into peptides significantly more rapidly than native hemoglobin, and a different set of peptides resulted. However, observation of RP-HPLC profiles showed that ovine hemoglobin, unlike bovine hemoglobin (mentioned in the literature), was not fully denatured by 5.3M urea.

Thirdly, native ovine hemoglobin, its apoglobins, and its peptides from pepsin digestion were tested for antimicrobial activity using the radial diffusion assay. The native hemoglobin tetramer displayed no activity at the highest concentration of 30mg/ml, but the separation of subunits at 0.5 to 2.0mg/ml provided moderate activity against *E.coli* and *S.aureus*. A greater proportion of the RP-HPLC fractions from the denatured hemoglobin pepsin digest were active towards *E.coli* and many were also more potent in comparison to those from the native digest. After further testing the denatured digest fractions against *S.aureus* and *C.albicans*, six candidates were selected for mass spectroscopy and MIC (Minimum Inhibitory

Concentration) testing based on their potency and reproducibility in RP-HPLC. Most of the peptides within these complex fractions were largely small random coils as desired. However, none of these fractions were highly antimicrobial, in fact, they had poor MICs ranging from 12mg/ml to 44mg/ml against the three test organisms.

It is recommended that further research be carried out focussing on the antimicrobial activity of a wider range of peptides with various secondary structures and peptide lengths. This would involve optimising digestion conditions and analysis of peptides from different degrees of hydrolysis. Synthetic peptides based on this information can be tested for their activities also. Then the feasibility of ovine hemoglobin peptides as components of antimicrobial treatments and products can be further investigated.

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Chapter 1

Project Introduction and Objectives

Many pathogenic microorganisms have become multi-drug resistant due to overuse and misuse of existing antibiotics. Drug-resistance is continually increasing due to human consumption, and the heavy usage in commercial industries, such as poultry farming, aquaculture (Marshall & Arenas, 2003) and agriculture (Dubin et al., 2005). The factors that have largely contributed to drug resistance are: overuse and misuse of existing antibiotics, bacterial mutations that arise with antibiotic use overtime, and the absence of new novel antimicrobials available on the market (Hancock, 1997). In fact, in the past 40 years the market has only seen two new structurally novel antibiotics (Coates & Hu, 2007). This is because drug companies realise that antibiotics are short-course treatments that cure infections (Coates & Hu, 2007) and that putting money and effort into new antibiotics that may only be effective for several years is less profitable than focussing on treatments for chronic disease, where patients are dependent on that treatment life-long.

Alarming statistics are constantly emerging indicating the seriousness of drug-resistant microorganisms such as the 'superbugs', Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant Enterococci (VRE) and multi-drug-resistant Pseudomonas (Coates & Hu, 2007). 'It is now estimated that about half of all *Staphylococcus aureus* strains found in many medical institutions are resistant to antibiotics such as methicillin' (Marshall & Arenas, 2003). Furthermore, approximately 70% of hospital acquired infections in the USA are resistant to at least one antibiotic (Clatworthy et al., 2007). Due to these facts, there is a justified concern that there will no longer be effective drugs available for the treatment of bacterial and fungal infections, resulting in an inevitable increase in deaths.

Antimicrobial peptides (AMPs) represent a possible alternative treatment to conventional antibiotics. AMPs are short sequences of amino acids found in the blood, skin, mucosa and secretions of almost all life-forms, which are part of the body's first line of defence against microbial infection (Parish et al., 2001), and have involvement in tissue homeostasis (Mak et al., 2000). AMPs isolated from various species have proven antimicrobial activity against a

wide range of bacteria, fungi and viruses (Beisswenger & Bals, 2005), and several are currently in drug trials. The most potent AMPs have MICs of 0.1 to 10ug/ml (Beisswenger & Bals, 2005; Hancock & Lehrer, 1998), which are competitive with some conventional antibiotics (Hancock, 1997; Hancock & Lehrer, 1998). Furthermore, these peptides possess many desirable characteristics for antimicrobial drug development: they are broad spectrum, rapidly kill microorganisms, and bacteria do not easily develop resistance to them (Hancock & Lehrer, 1998); most cationic peptides do not induce resistant mutants even after 20 passages on an antibiotic concentration close to the MIC (Hancock, 1997). It is believed that these desirable traits of AMPs are attributed to their physical mechanism of killing, that is, they typically target and disrupt cell membranes.

It has been known for some time that blood is a source of antimicrobial molecules. Types of white blood cells (WBCs) known as granulocytes possess granules in their cytoplasm, which contain an array of antimicrobial molecules, such as defensins and cathelicidins (AMPs), lysozyme, lactoferrin, cathepsin G, elastase, bactericidal permeability increasing protein (BPI), collagnease and digestive enzymes (Kaiser, 2010). Interestingly, in the 1950s it was reported for the first time that hemoglobin, the oxygen carrier in the red blood cells (RBCs), may also exert antimicrobial activity (Hobson & Hirsch, 1958). However, reports of the first antimicrobial hemoglobin-derived peptide only occurred within the last thirteen years (Fogaca et al., 1999). These peptides reportedly possess antimicrobial properties far greater than that of the intact parent protein. In particular, there is evidence to suggest that short random coiled peptides from enzymatic digestion of hemoglobin are extremely potent, with MICs in the range of 1-10ug/ml (Nedjar-Arroume et al., 2008). Furthermore, shorter peptides reduce production costs (Falla & Zhang, 2004; Strom et al., 2003).

Most of the literature currently available on antimicrobial activity of hemoglobin derived peptides and the intact protein focus on peptides from cattle and humans. There are no known publications on peptides from sheep hemoglobin. This research aims to address this.

The sheep meat export market is a hugely important source of income for New Zealand, earning an all-time high of $2.9 billion in 2011 (Statistics New Zealand, 2012). New Zealand is responsible for about 55% of the international sheep meat trade and 75% of lamb meat trade (McDermott A., 2008), slaughtering 31.1 million sheep in 2011 (Statistics New Zealand,

2012). This results in hundreds of thousands of litres of ovine blood every year from which currently only low value products are made, that is, blood meal to be used as an animal feed ingredient (mainly in the pork industry) (Alsweiler, 2011), or as a fertiliser. This sells at $900 to $1200 NZD per metric tonne depending on the market and product specifications (Alsweiler, 2011). It is proposed that this blood could potentially be used to create products of greater value.

This research aims to produce antimicrobial peptides from the hemoglobin of sheep blood that results from slaughter, and test their antimicrobial potency, as well as that of the intact protein and its subunits. These peptides/proteins could be used in the treatment of human and animal infections. They could also be used as biopreservatives in chilled lamb products, providing extended shelf life. Such applications could be hugely beneficial to human and animal health and would result in further wealth in the New Zealand sheep meat industry.

The objectives of this research are to:

1. Identify a method of isolating hemoglobin from fresh whole ovine blood.
2. Identify the conditions required to separate globin chains and digest native hemoglobin to obtain short random coiled peptides.
3. Purify globin chains into alpha and beta fractions, and purify the peptide digestion hydrolysate.
4. Test the antimicrobial activity of native hemoglobin, apoglobins and peptides against common laboratory organisms.
5. Identify a possible relationship between hemoglobin peptide characteristics and potency towards microorganisms.

Chapter 2

Literature Review

2.1 Introduction

This chapter is a review of the current knowledge on antimicrobial peptides and their potential use as novel antimicrobial agents. First, the characteristics of AMPs were investigated. AMPs are produced by all life forms as a natural defense mechanism against microbial invasion. Aspects of their structure that enables this antimicrobial activity are discussed, as well as structural classifications.

Next, it was necessary to distinguish and define two broad classes of antimicrobial peptides: classical AMPs and AMPs derived from functional proteins. Features of the most characterised classical mammalian peptides, the defensins and cathelicidins, and prokaryotic peptides are described.

A brief history of bioactive peptides generated from functional proteins is discussed, as knowledge of these bioactive peptides was a precursor to the discovery of AMPs from functional proteins.

The killing or inhibition of microbial growth by AMPs may be exerted by direct action against microbial membranes and intracellular components, or through mediating activities of effector cells in the immune system. Therefore, the roles of AMPs in both the innate and adaptive immune systems were considered.

A significant section of this review covers the structure of hemoglobin, and the formation of its peptides *in vivo* and *in vitro*. This includes conditions, chemicals and enzymes necessary to achieve AMP generation *in vitro*, and the proteolytic degradation kinetics by pepsin. Furthermore, the structure of hemocidins (AMPs from heme-containing proteins) and their antimicrobial mechanisms were examined, as well as the effect of common antagonistic factors on peptide antimicrobial function.

Lastly, the current position and potential of AMPs as therapeutic drugs and biopreservatives was explored. The pros and cons of AMP usage, success of companies with AMP products in clinical trials, and the applications of ovine AMPs were discussed.

2.2 Properties of Antimicrobial Peptides

Unlike conventional antibiotics, which are chemical compounds produced by microorganisms, AMPs are short sequences of amino acids that are produced in almost all life-forms. These peptides are a component of the body's first line of defence against microbial infection, and possess the ability to kill microorganisms or inhibit their growth (Parish et al., 2001), typically via their interactions with cell membranes. Many AMPs also have significant roles in mediating immunity, inflammation and wound repair in the innate immune system (Hiemstra et al., 2004). For this reason they are often called host defense peptides.

AMPs are considered to possess great potential as antimicrobials. They exert broad-spectrum activity and may be active against Gram positive and Gram negative bacteria, yeasts, fungi, and certain enveloped viruses and protozoa (Giuliani et al, 2007). The MICs (minimum inhibitory concentration) of the more effective AMPs are comparable to traditional antibiotics, at 0.1-10ug/ml (Beisswenger & Bals, 2005; Hancock & Lehrer, 1998). They also act rapidly in response to infection and it has been shown that unlike many antibiotics, microorganisms do not easily develop resistance to them.

The first AMPs were isolated in the 1970s from amphibians, insects and plants (Nedjar-Arroume et al., 2008). So far, over 1,200 antimicrobial peptides have been described (Lai & Gallo, 2009), with more than half of these isolated from insects (Marshall & Arenas, 2003).

2.3 Structures of Antimicrobial Peptides

AMPs are typically less than 100 amino acids long (Ganz, 2003; Jones et al., 2005) and possess particular structural features that allow for their interaction with cell membranes. Most are cationic peptides rich in the basic residues lysine, arginine and histidine, whilst few are anionic, rich in aspartic and glutamic acids (Brogden, 2005). Neutral AMPs also exist.

Cationic peptides are usually comprised of at least 50% hydrophobic residues (Hancock & Diamond, 2000) and are amphipathic, having hydrophilic and hydrophobic residues on

opposite sides of the peptide axis (Brogden, 2005). Alanine, leucine, phenylalanine and tryptophan contribute to their hydrophobic nature (Brogden, 2005). The hydrophobic to basic residue ratio of an AMP is typically 1:1 or 2:1 (Brogden, 2005).

AMPs are a very structurally diverse group of molecules that can be grouped according to several factors, such as secondary structure, net charge, amino acid composition and size. The following groupings classify AMPs on the basis of their 3D structure and overall composition:

- Group 1: linear peptides with an alpha-helical structure.
- Group 2: beta-sheet structures stabilised by disulphide bridges.
- Group 3: peptides with predominance of one or more amino acids.
- Group 4: peptides with loop structures (Beisswenger & Bals, 2005; Koczulla & Bals, 2003).

Examples of peptides for each of these categories can be seen in Table 2.1.

Table 2.1 – Structural groupings of AMPs (Koczulla & Bals, 2003).

Peptide	Species, organ	Activity
Group I: Linear, α-helical peptides without cysteines		
Bombinins	Frog, skin	Antimicrobial
Cecropins	Insects, haemocytes, sperm	Antimicrobial
LL-37	Human, neutrophils, epithelial cells	Antimicrobial, chemoattractant
Magainins	Frog, skin	Aantimicrobial
Styelins	Tunicates, haemocytes	Antimicrobial
Clavanins	Tunicates, haemocytes	Antimicrobial
Melittin	Bee, venom	Antimicrobial
Group II: β-sheet structures stabilised by two or three disulphide bridges		
Protegrin	Pig, intestine	Antimicrobial
Tachyplesins	Horseshoe, haemocytes	Antimicrobial
Defensins	Vertebrates, immune cells, epithelia	Antimicrobial, chemoattractant
Insect defensins	Insects, haemocytes	Antimicrobial
θ-Defensins	Monkeys, neutrophils	Antimicrobial
Plant defensins	Plants, seeds, leaves	Antimicrobial
Drosomycin	Insects, haemocytes	Antimicrobial
Group III: Peptides with a predominance of one or more amino acids		
PR-39	Pig, intestine, neutrophils	PR-39, angiogenesis, wound healing
Bac5, Bac7	Cow, neutrophils	Antimicrobial
Drosocin	Drosophila,[a] haemolymph	Antimicrobial
Metchnikowin	Drosophila,[a] haemolymph	Antimicrobial
Group IV: Peptides with loop structures		
Bactenecin	Cow, neutrophils	Antimicrobial
Ranalexin	Frog, skin	

a *Drosophila melanogaster*, the fruit fly.

2.4 Classical AMPs versus AMPs Derived from Functional Proteins

There are two broad groups of AMPs based on their origin *in vivo*. These are the classical AMPs, and those that arise from cleavage of larger functional proteins. It should be noted however, that both groups require proteases to cleave precursors into final active products. Literature available on classical AMPs is numerous, whereas there is much less documentation on peptides derived from already functional proteins.

2.4.1 Classical AMPs

AMPs in this class are encoded by specific genes in an organism's genome. Families of AMP genes are present in a clustered fashion in the genome, and the chromosomal location of many AMP genes is conserved from one species to another (Beisswenger & Bals, 2005). The peptides may be synthesised ribosomally, as in the case of eukaryotes, or either ribosomally or non-ribosomally in bacteria. In mammals, these peptides are synthesized in cells that are prone to microbial invasion, such as epithelial cells in mucosal regions of the body and in phagocytic cells (Jones et al., 2005). Here, they are expressed constitutively or induced in response to infection. In the case of an induced expression, when infection occurs, interaction of a microbe, microbial macromolecules (PAMPs: Pathogen associated molecular patterns) and/or proinflammatory cytokines (IL-1beta, TNFalpha)(Ganz, 2003; Yang & Oppenheim, 2004) with specific host effector cell receptors causes the activation of signalling pathways resulting in the expression of AMPs (Beisswenger & Bals, 2005).

As shown by Figure 2.1, classical AMPs are expressed as a translational product consisting of 'pre', 'pro' and 'mature' regions and are known as prepropeptides (Hancock & Diamond, 2000). They can be stored in cells as inactive propeptides, or mature 'active' C-terminal peptides (Beisswenger & Bals, 2005). The C-terminal region may contain one or multiple copies of the antimicrobially active peptide, which are released upon proteolytic cleavage (Ganz, 2003). The pre-region is an N-terminal signal sequence for targeting the endoplasmic reticulum (Beisswenger & Bals, 2005; Hancock & Diamond, 2000). The pro-region is often anionic (Beisswenger & Bals, 2005) and is thought to protect the cationic mature peptide

from interaction with the host cell membrane and ensures correct C-terminal mature peptide folding occurs (Hiemstra et al., 2004). Furthermore, it may have a role in intracellular trafficking (Beisswenger & Bals, 2005). Once the C-terminal segment is cleaved to give active peptide(s), the peptides can then be released systemically or locally (Jones et al., 2005).

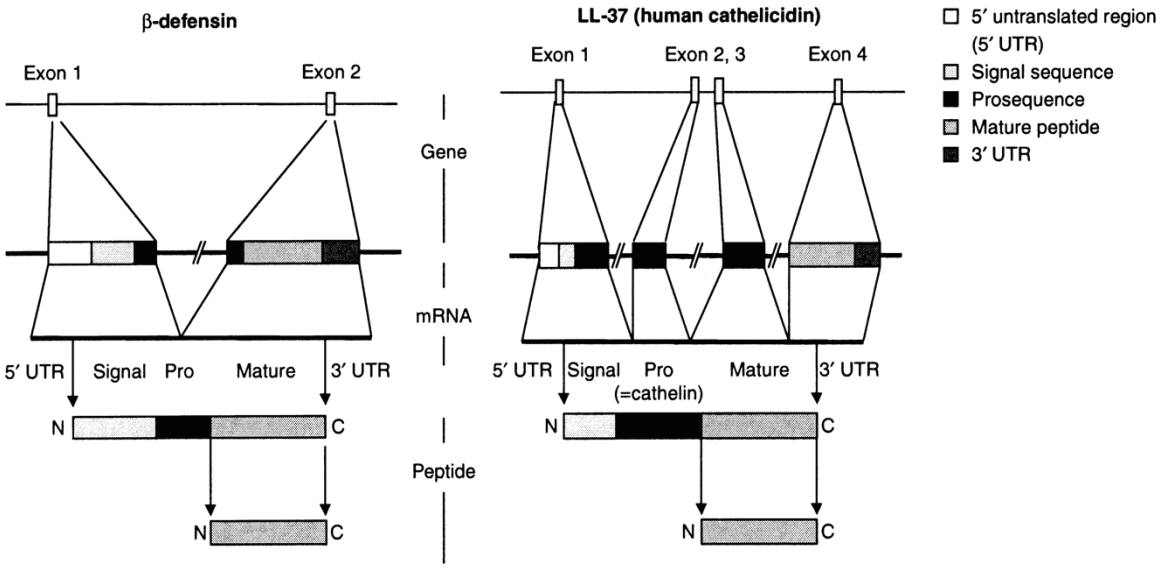

Figure 2.1 - Expression of classical gene encoded AMPs. Gene transcription followed by translation to give a peptide consisting of pre, pro, and mature peptide regions. Proteolysis results in activation of the mature peptide region (Koczulla & Bals, 2003).

2.4.1.1 Classical AMPs from Eukaryotes

The two widely studied classical eukaryotic AMP classes are the defensins and cathelicidins.

2.4.1.1.1 Defensins

Defensins are cationic AMPs that contain six cysteine residues (Beisswenger & Bals, 2005) arranged into three intramolecular disulfide bridges within a beta-sheet structure (Hiemstra et al., 2004). They range from 29 to 47 amino acid residues (Ganz, 2003), and are classified into the subgroups alpha, beta and theta-based on the location and pairing of the the cysteine residues (Beisswenger & Bals, 2005). The alpha defensins have cysteine (C) pairings of C1-C6, C2-C2 and C3-C5, whilst beta defensins have C1-C5, C2-C4 and C3-C6 (Beisswenger & Bals, 2005).

The alpha defensins, HNP1 to 4 (human neutrophil peptides), are stored at high concentrations as fully processed mature peptides (Gallo & Nizet, 2003) in the azurophilic granules of neutrophils (Hiemstra et al., 2004). These peptides are involved in oxygen-independent killing of microbes (Beisswenger & Bals, 2005) and are constitutively expressed (Hancock & Lehrer, 1998). HD-5 and HD-6 (human defensins) are expressed by specialised Paneth cells in the small intestine (Gallo & Nizet, 2003). The expression of these is induced by infection or inflammation (Hancock & Lehrer, 1998).

Four human beta defensins, HBD-1-4, have been characterised in detail. They can be found at a wider variety of surfaces, inside and outside the body, and in monocytes/macrophages and dendritic cells (Hiemstra et al., 2004). HBD-1 is expressed constitutively in the epithelial cells of the urinary and respiratory tract, whilst HBD-2 is found in places such as inflamed skin, and the gastrointestinal and the respiratory tract (Beisswenger & Bals, 2005). HBD-2-4 are inducible (Hiemstra et al., 2004).

Little is known about the last group of defensins, the theta defensins. Three peptides, rTD-1-3, have been isolated from the neutrophils of the Rhesus monkey and have a circular molecular structure (Beisswenger & Bals, 2005; Koczulla & Bals, 2003). There is currently no data on their regulation or the presence in tissues (Ganz, 2003; Koczulla & Bals, 2003).

2.4.1.1.2 Cathelicidins

Cathelicidins have highly conserved signal and N-terminal cathelin-like sequences (seen in Figure 2.1), and an extremely variable C-terminal sequence, which is activated when proteolytically cleaved. The cathelin-like domain is so named as it resembles a protein isolated from porcine neutrophils, which acts as an inhibitor of the cysteine-protease, cathepsin L. This domain possesses some antimicrobial activity (Gallo & Nizet, 2003; Lai & Gallo, 2009).

The C-terminal peptide is responsible for the main broad spectrum antimicrobial activity, synergy with other AMPs, and activation of host cells (Gallo & Nizet, 2003). It may be of a linear, beta-sheet or alpha-helical structure due to the diversity of the C-terminal sequence within and between species (Lai & Gallo, 2009).

A variety of cathelicidins are produced by birds, fish, snakes and mammals (Lai & Gallo, 2009), ranging from 12-80 plus amino acids (Beisswenger & Bals, 2005; Koczulla & Bals, 2003). However, there is only one known cathelicidin produced in humans, LL-37, and it is cationic and alpha helical (Lai & Gallo, 2009). This is mainly found stored as inactive precursors in neutrophils, where it is proteolytically cleaved and activated by proteinase 3, and then released (Hiemstra et al., 2004). LL-37 is also found in other granulocytes, such as NK-cells and mast cells, and is expressed by epithelial cells in the skin, lungs, gut, mammary gland and epididymis (Lai & Gallo, 2009). At the skin surface, the peptide is further processed by serine proteases into various forms (Lai & Gallo, 2009).

2.4.1.2 Classical AMPs from Prokaryotes

Peptides that are produced ribosomally by microorganisms, and inhibit or kill microorganisms other than the producing strain, are termed bacteriocins (Cotter et al., 2005). Bacteriocins can be cationic, neutral or anionic in charge and greatly vary in size (Marshall & Arenas, 2003), upon which they are typically grouped (Cotter et al., 2005). The sub-groups include: thiolbiotics, lantibiotics, microcins and colicins (Marshall & Arenas, 2003). Some are said to have a narrow spectrum, while others are broad spectrum in activity (Cotter et al., 2005).

A prominent example of a bacteriocin is the lantibiotic, nisin (Koczulla & Bals, 2003). Nisin has a high affinity for lipid II, a membrane-bound peptidoglycan precursor, and the ability to create pores in cell membranes (Koczulla & Bals, 2003). This peptide, produced by the lactic acid bacterium *Lactococcus lactis* subsp. *Lactis* is the only bacteriocin approved for use as a food preservative in certain products (Jones et al., 2005).

Prokaryotic AMPs may also be produced non-ribosomally.

2.4.2 Bioactive Peptides Derived from Functional Proteins

Unlike classical AMPs, this class of peptides is not the result of specialised gene expression. Instead, peptides are produced due to proteolytic degradation of existing proteins that possess a primary function. The resulting peptides are sometimes called cryptides (Nedjar-Arroume et al., 2008).

It should be noted that bioactive peptides proteolytically derived from functional proteins have been known about for several decades (Karelin et al., 1998). These were first identified as a result of digesting functional proteins *in vitro* with various proteases. Later, biologically active peptides from functional proteins were also isolated *in vivo*.

In 1941, the first observation of bioactive peptides from functional protein was made, when blood plasma proteins were treated with pepsin (Karelin et al., 1998). The resulting hydrolysate possessed the ability to cause histamine release from mast cells. Since then fragments from serum albumin, gluten, cytochrome c, hemoglobin and milk proteins (casein, alpha lactalbumin, beta lactoglobulin and lactoferrin) have been discovered to carry out various biological functions (Dubin et al., 2005; Karelin et al., 1998), including opioid activity.

2.4.2.1 AMPs Derived from Functional Proteins

One of the first known human AMPs produced as a result of proteolysis of a functional protein was from human BPI protein (bactericidal/permeability increasing protein) (Mak, 2008). BPI is a ~50kDa, 456 residue, lipid-binding protein that is produced by neutrophil precursors in bone marrow and stored in their primary granules. The protein itself possesses antibacterial activity against Gram negative bacteria (Elsbach, 1998; Mak, 2008). It is understood that the carboxy-terminal of the protein has no antimicrobial activity, whilst 21-25kDa fragments from the amino-terminal do (Weiss et al., 1978). A 21kDa cationic BPI fragment from the amino-terminal, named $rBPI_{21}$ by Xoma, has antibacterial, antifungal, anti-endotoxin activity, synergism with antibiotics, and is currently in clinical trials (Campbell & Reece, 2005). This will be further discussed later on.

Another protein that has antimicrobial domains is human cathepsin G. This 23kDa lysosomal protein from human neutrophils has serine protease activity (Mak, 2008). In 1990 Bangalore et al. (1990) discovered that the antimicrobial activity of cathepsin G is independent of its primary enzymatic ability. Treatment of the protein with an irreversible serine protease inhibitor had no effect on its antimicrobial activity. Furthermore, digestion of cathepsin G into fragments with clostripain (endoproteinase arg-C) resulted in a loss of proteolytic activity but not antimicrobial activity (Bangalore et al., 1990). It was later found that cathepsin G has at least three antimicrobial domains, with activity against Gram negative bacteria, and some of its peptides also have activity towards Gram positive bacteria (Mak, 2008).

There are numerous examples of antimicrobial peptides derived from milk proteins, but a prominent example is those from the iron-binding protein, lactoferrin. Lactoferrrin is an 80kDa globular glycoprotein consisting of alpha helices, which is part of the innate defense at mucosal regions (Farnaud & Evans, 2003). Therefore, it is also present in tears, saliva and nasal excretions, but is present in its highest concentration in colostrum. It was thought that its ability to sequester iron from the environment was responsible for inhibition of microbial growth. It was later found that the protein's antimicrobial action was independent of its iron-binding function, and instead due to its ability to damage outer bacterial membranes. A class of antimicrobial peptides called lactoferricins was generated by peptic hydrolysis of lactoferrin. These linear cationic 15-18 residue peptides were derived from the N-terminal of lactoferrin, and possessed greater antimicrobial activity than the parent protein itself. Later, lactoferricin peptides were also isolated from *in vivo* sources such as in the stomach and in mucosal secretions, where lactoferrin is likely digested with gastric enzymes such as pepsin. Although the mechanism of action of the lactoferricin class is unknown, these peptides have the ability to kill a broad range of microbes, such as bacteria, fungi and viruses (Mak, 2008).

Other antimicrobial peptides that arise from limited proteolytic degradation of milk proteins include casosidins and isracedin from casein, as well as peptides from alpha-lactalbumin and beta-lactoglobulin (Mak, 2008). Peptides derived from heme-containing proteins will be discussed in detail later in this chapter.

2.5 AMP Modes of Action

It is well accepted that AMPs can interact directly with bacterial membranes, disrupting membrane integrity or can act without membrane disruption, binding intracellular targets. They can inhibit cell growth or kill cells i.e. they are bacteriostatic or bactericidal.

The selectivity of AMPs for prokaryotic cells rather than eukaryotic cells is based on their different membrane properties. Bacterial membranes are rich in anionic phospholipids allowing for essential electrostatic interactions with cationic peptides. Conversely, animal cell membranes are rich in neutral phospholipids, and contain cholesterol (Ganz, 2003). It is thought that since cholesterol condenses the membrane, this may also prevent AMPs from entering eukaryotic cell membranes (Lai & Gallo, 2009). These characteristics explain why far greater concentrations of AMPs are required to kill eukaryotic cells as opposed to bacterial cells (Ganz, 2003).

Again, the different characteristics of Gram positive versus Gram negative bacterial membranes strongly influence the ability of antibiotics and AMPs at entering cells, and therefore determine their effectiveness. Gram positive bacteria have a thick outer peptidoglycan layer (includes teichoic acid and lipoteichoic acid) and an inner cytoplasmic membrane, whereas Gram negative bacteria have an outer membrane and a cytoplasmic membrane separated by periplasm that includes peptidoglycan. The outer membrane of Gram negative bacteria includes porin proteins, which are responsible for the uptake and restriction of small molecules, such as antibiotics. Gram positive bacteria lack this protein. This is a primary reason as to why it is more difficult for antibiotics to gain access to a Gram negative cell, in comparison to a Gram positive cell (Jones et al., 2005).

Furthermore, AMPs use a different mechanism to enter bacterial cells than conventional antibiotics. The Gram negative outer membrane has a LPS (lipopolysaccharide) monolayer that cationic AMPs utilise to enter the cell by the mechanism of self-promoted uptake. The LPS monolayer has strong negative charges that are partly neutralised/stabilised by the presence of bound divalent cations- particularly Ca^{2+} and Mg^{2+}. Cationic AMPs can interact with the LPS charges, displacing the divalent cations and resulting in destabilised areas in the outer membrane. AMPs can then travel through the disrupted regions of the outer

membrane. (Giuliani et al., 2007; Jones et al., 2005). In the case of Gram positive bacteria, initially AMPs will electrostatically bind to teichoic acids (TA) or lipoteichoic acids (LTA) instead (Lai & Gallo, 2009). Once the cytoplasmic membrane is reached, the peptides can associate with the outer cytoplasmic monolayer and insert into the membrane.

There are three main models proposed for cytoplasmic membrane disruption by AMPs: carpet, barrel-stave, and toroidal-pore mechanisms (Brogden, 2005). These can be seen in Figure 2.2. The mechanism that is utilised is based on factors, such as peptide amino acid sequence, peptide concentration, membrane phospholipid characteristics (Giuliani et al., 2007), and the size of the membrane potential gradient (Jones et al., 2005).

In the carpet-like mechanism, peptides are electrostatically attracted to the negative charges on the phospholipid head groups of the outer monolayer and align themselves parallel to the membrane surface (Brogden, 2005). When a high density of peptides cover the surface, the membrane experiences curvature strain due to phospholipid displacement/destabilisation. This causes changes in the membranes fluidity and/or reduces the membrane barrier function, leading to membrane disruption. Unlike other methods of membrane disruption, the carpet mechanism does not involve the formation of channels or pores, and peptides do not necessarily enter the membrane (Giuliani et al., 2007).

The barrel-stave mechanism involves a small number of peptides inserting perpendicularly into the membrane to form transmembrane pores. First, the hydrophobic regions of the peptides insert into the bilayer aligning with the hydrophobic core. At a critical concentration peptides self-aggregate, sheltering hydrophilic residues as they enter further into the membrane hydrophobic core (Giuliani et al., 2007). The result is a transmembrane pore where the peptides hydrophobic residues align with the hydrophobic membrane core, and the lining consists of hydrophilic residues (Brogden, 2005; Giuliani et al., 2007). Few peptides have been found that utilise this mechanism (Giuliani et al., 2007; Lai & Gallo, 2009).

Unlike the barrel-stave mechanism, in the torodial pore mechanism the transmembrane pores that are created are lined with both peptides and the phospholipid heads of the membrane. As peptides insert into the membrane they cause the lipid monolayers to bend inwards continuously through the pore so that the polar phospholipid heads face the polar

regions of the peptides (Brogden, 2005; Giuliani et al., 2007). The association between the lipids and peptides stabilises the high positive density of charge in the pore.

All these membrane disruptive methods require a sufficient peptide to lipid ratio to occur. The mechanisms discussed are assumed to cause microbial death, but death can also occur because membrane disruption creates an entrance for other lethal molecules, which act intracellularly i.e. lysozyme, lactoferrin, secretory proteinase inhibitor and elafin (Mak et al., 2004). The presence of molecules with different modes of action may allow for a synergistic effect. This is where the overall activity of the peptide mixture is greater than the sum of their individual antimicrobial activities. Many peptides have this ability to carry out this activity with other AMPs or antibiotics.

Furthermore, there is evidence to show that microbial cells can be killed without membrane disruption by AMPs that utilise unique methods of membrane penetration and directly bind to intracellular targets (Brogden, 2005; Giuliani et al., 2007; Lai & Gallo, 2009). The intracellular targets include: inhibition of cell wall, nucleic-acid and protein synthesis, binding to nucleic-acids, flocculation of intracellular contents and inhibiting enzyme activity (Brogden, 2005). Also, a single AMP may possess more than one of the above cell targets, which can partly account for the lack of resistance developed by microbes (Berg et al., 2002).

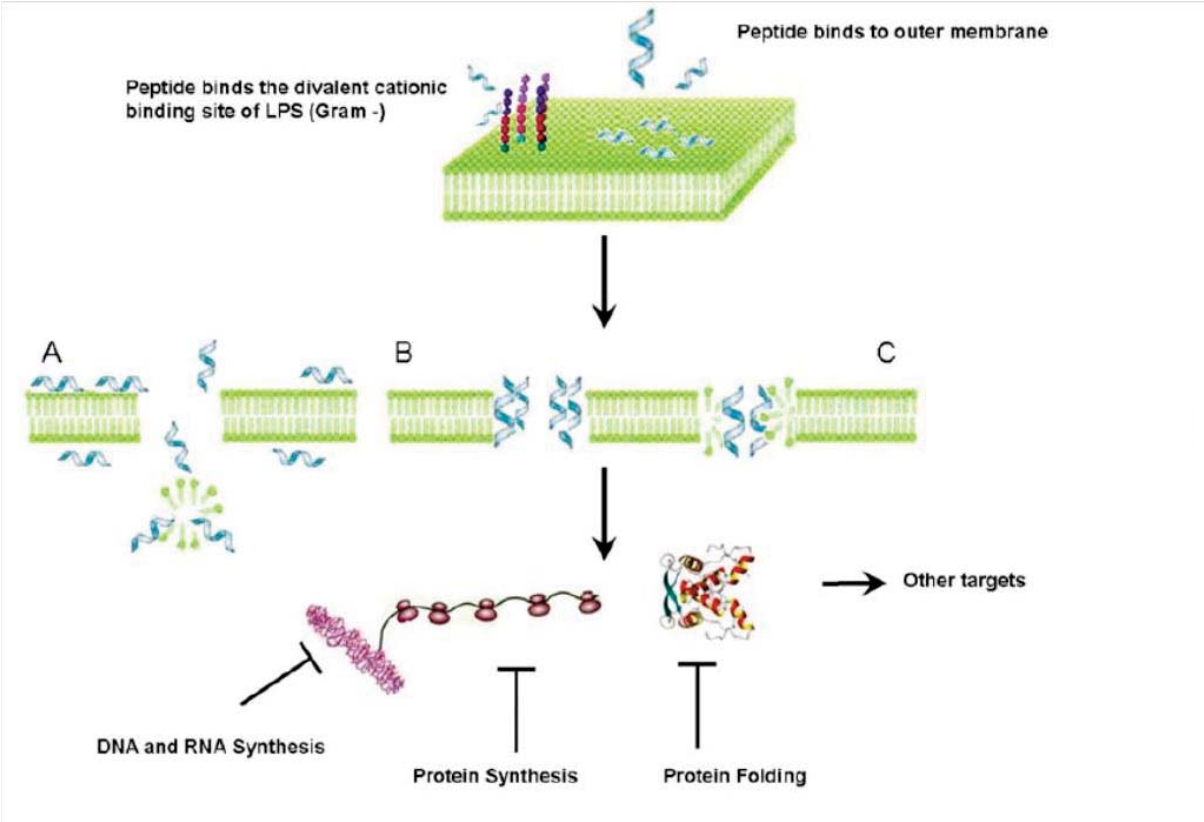

Figure 2.2 - Membrane disruptive mechanisms and intracellular targets of AMPs. (A) Carpet mechanism, (B) Barrel-stave mechanism, (C) Torodial pore mechanism (Giuliani et al., 2007).

2.6 Immunity

2.6.1 The Role of AMPs in the Innate Immune System

Active AMPs are chemoattractants for specific receptors on host defense cells in the immune system. They directly recruit effector cells, such as neutrophils, monocytes and macrophages to the infection site by chemotaxis. For example, cathelicidin LL-37 is chemotactic for neutrophils, monocytes and T cells (Ganz, 2003; Lai & Gallo, 2009). Other AMPs may induce the expression of chemokines or cytokines as an indirect method for recruiting effector cells (Lai & Gallo, 2009). Some AMPs (e.g. human beta defensins 1 and 2) are chemoattractants for mast cells, which degranulate releasing products such as histamine and prostaglandin (Beisswenger & Bals, 2005). These products have the ability to increase the permeability of the capillaries, resulting in an increased neutrophil influx to the infection site (Jones et al.,

2005). Furthermore, neutrophil degranulation at the infection site releases more AMPs and, hence, further recruitment results.

AMPs can also enhance or reduce gene expression. There is evidence that LL-37 can enhance gene expression by macrophages, upregulating chemokines and their receptors. This can result in an increase of recruited effector cells to the inflammatory site. Other AMPs, HNP1-HNP3, increase the production of proinflammatory cytokines (tumor necrosis factor (TNF)alpha and interleukin (IL)-1) and decrease IL-10 production from monocytes, amplifying inflammatory responses (Jones et al., 2005). Moreover, upregulation of adhesion molecules on the surface of phagocytes leads to enhanced phagocytosis.

AMPs have roles in wound healing. Cathelicidins and defensins have been found to be present in high concentrations at wound edges (Lai & Gallo, 2009). LL-37 is found in very high concentrations in injured skin, and is involved in wound closure and re-epithelialisation of human skin (Beisswenger & Bals, 2005; Lai & Gallo, 2009). It has also known that the inhibition of LL-37 prevents skin re-epithelialisation (Beisswenger & Bals, 2005). Furthermore, LL-37 has a particularly important role in the proliferation stage of wound healing and induces angiogenesis (the formation of new blood vessels by vascular endothelial cells), necessary in cutenous wounds (Lai & Gallo, 2009). HBD2 has a similar role, it 'stimulates migration, proliferation and tube formation of endothelial cells in wounds, leading to accelerated wound closure' (Lai & Gallo, 2009).

AMPs also have the ability to neutralise the activity of endotoxin, LPS (lipopolysaccarides), from Gram negative bacteria, and LTA (lipoteichoic acids), from Gram positive bacteria (Yang & Oppenheim, 2004). When bacteria are killed by AMPs, phagocytes, and the complement system of the innate immune system, LPS and LTA are released (Jones et al., 2005). When LPS or LTA are present in the body, they react with macrophage receptors, which induce the production of proinflammatory cytokines, particularly tumor necrosis factors (TNFs) (Hancock & Diamond, 2000). Therefore, when LPS of LTA concentration is high, the proinflammatory cytokine concentration is high, and this can lead to sepsis (systemic inflammatory response syndrome) (Hancock & Diamond, 2000). As mentioned earlier, the detection of PAMPs (Pathogen-Associated Molecular Patterns) such as LPS or LTA, induces AMP synthesis and/or release. The AMPs can then bind to LPS or LTA, inhibiting the ability to interact with macrophage receptors, limiting proinflammatory cytokine production, and

hence decreasing the likelihood of a sepsis response (Hancock & Diamond, 2000). Generally, cathelicidins are better in doing so than defensins. Ovine peptide, SMAP-29, has been shown to neutralise LPS *in vitro* and *in vivo* (Jones et al., 2005).

In addition to their ability to neutralise LPS and LTA, AMPs can also directly inhibit the expression of proinflammatory cytokines from macrophages (Jones et al., 2005). It was found that LL-37 suppresses the release of TNF from human macrophages and monocytes in the presence of LPS or LTA (Lai & Gallo, 2009).

Other important roles that AMPs have in the innate immune system include: regulation of the complement system by enhancing or suppressing activation of the classical pathway and suppressing anti-inflammatory or immunosuppressive mediators.

2.6.2 The Role of AMPs in the Adaptive Immune System

If an infection overcomes the innate immune system, the adaptive immune system comes into action. It is thought that AMPs play a role here in the recruitment of antigen-presenting dendritic cells (Ganz, 2003) and T cells (Hiemstra et al., 2004) to the infection site.

Immature dendritic cells (iDCs) utilise their surface and intracellular molecules to uptake and process antigens at the infection site. It is necessary that iDCs migrate to the infection site where the microbial antigen concentration is high enough for processing. Certain AMPs can chemoattract iDCs directly to the infection site for this process to occur, whilst some AMPs chemoattract monocytes, which are precursors to dendritic cells.

After antigen processing, iDCs mature to become mature dendritic cells (mDCs). This is induced by proinflammatory cytokine production (IL-1beta and TNFalpha), which is indirectly induced by AMPs. These cells then have the ability to migrate to the regional lymph nodes where they present the processed antigenic peptide complex on their surface to the naive T-cells. mDCs have a chemokine receptor, CCR7, on their surface especially for this trafficking. Furthermore, the expression of CCR7 upregulates surface co-stimulatory molecules on the mDC surface, which stimulate T-cell activation and expansion.

Antigen-specific effector T-cells and antibodies are generated after the activation and expansion of T and B lymphocytes, respectively. Some AMPs are also chemoattractants for T-cells (Jones et al., 2005).

The fact that some AMPs have the ability to bind to the receptors on immature dendritic cells and memory T cells (e.g human beta defensins 1 and 2) is evidence that these peptides provide a link between the innate and adaptive immune systems (Beisswenger & Bals, 2005). Table 2.2 and Figure 2.3 provide a summary of the major roles of AMPs in the immune system.

Table 2.2 – Roles of specific AMPs in the immune system (Hancock & Diamond, 2000).

Peptide[b]	Effect	Possible role in immunity	Refs
LL-37; PR-39; TAP; LAP; α- and β-defensins	Induction during inflammation	Synthesis triggered by situations that might involve infection	22,28–34
LL-37; **CP-28**	Directly stimulate transcription of >30 genes in macrophage cell line	Modulation of the activity of phagocytic cells	45
CAP11; Magainin 2	Promote histamine release from mast cells	Stimulate increase in blood vessel permeability	9,10
α-defensins	Induce IL-8 in airway epithelial cells	Recruitment of neutrophils	53
PR-39	Chemotactic activity for neutrophils	Increase phagocytic activity	10,47
LL-37; HNP-1, -2 and –3; histone H2B fragments	Induction in wounds and blisters	Wound healing (?)	9,10
Guinea-pig defensins	Increased expression of CD11b,c and ICAM-1	Increase adherence of neutrophils	9,10
Rabbit defensins NP-1,2	Promote non-opsonic phagocytosis by macrophages	Bacterial clearance	45
CA(1–8)M(1–18)	Influence signalling pathways in macrophages; induce iNOS synthesis	Bacterial clearance; activation of macrophages	51
Defensin I and II	Inhibit fibrinolysis by tissue plasminogen activator	Limiting spread of infection	9,10
Defensin; SB-37; **Shiva**, **Vishnu**	Stimulate mitogenic effect for fibroblasts and epithelial cells; stimulate fibroblast growth	Wound healing	46
PR-39	Induce cell surface matrix proteogylcans, syndecans 1 and 4	Wound healing	47
Histatin 3; pro Bac 7	Inhibition of furin proprotein convertase/cathepsin L protease	Inhibit tissue injury during inflammation	50
BMAP-27; BMAP-28; **CA(1–8)M(1–18)**; Lactoferricin	Apoptosis in the U937 and RAW264.7 macrophage lines and in *in vitro*-activated human lymphocytes	Elimination of cells with intracellular bacteria, virus-infected cells and cancer cells	10,49,52
Defensins	Attenuates steroidogenesis	Blunt the release of immunosuppressive cortisol during stress from infection; upregulated tissue inflammation	4,9
CAP11	Increased neutrophil adhesion; inhibits phagocytosis of opsonized zymosan particles	Modulation of neutrophil functions	10
ProBac 7; defensins	Chemotactic activity for monocytes	Recruitment of monocytes, which differentiate into inflammatory macrophages	9,10,49
CAP18(106–137); LL-37; **CP-28** and many other peptides	Neutralize LPS (endotoxin) responses in macrophages and animal models	Antisepsis; feedback inhibition of endotoxin responses	10,38,39
Many peptides	Neutralize LTA responses in macrophages	Antisepsis	45
LL-37; **CP-28**	Neutralize CpG responses in macrophages	Antisepsis	c
LL-37; **CP-28**	Selectively suppress expression of >40 LPS-induced genes in a macrophage cell line	Antisepsis; reduce cytokine expression	45
HNP-1, -2; LL-37	Chemotactic activity for T cells	Recruit T helpers and initiate cellular immune responses	48
Nisin	Induce increase in CD4 and CD8 lymphocytes in mice on short-term administration to diets	Increase cellular immunity and T-cell help	9
Defensins; HNP-1, -2 and -3	Enhancement of IFN-γ, IL5, K6, K10 and proliferative responses by T-helper cells cytokine secretion, increased systemic IgG but not IgA	Promotion of acquired systemic immune defenses (not mucosal immune defences)	52

[a]Abbreviations: iNOS, intracellular nitric oxide synthase; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LAP, lingual antimicrobial peptide; LTA, lipoteichoic acid; LPS, lipopolysaccharide; TAP, transporters associated with antigen processing.
[b]Natural peptides are in normal typeface whereas synthetic peptides are indicated in bold.
[c]R.E.W. Hancock and M.G. Scott, unpublished.

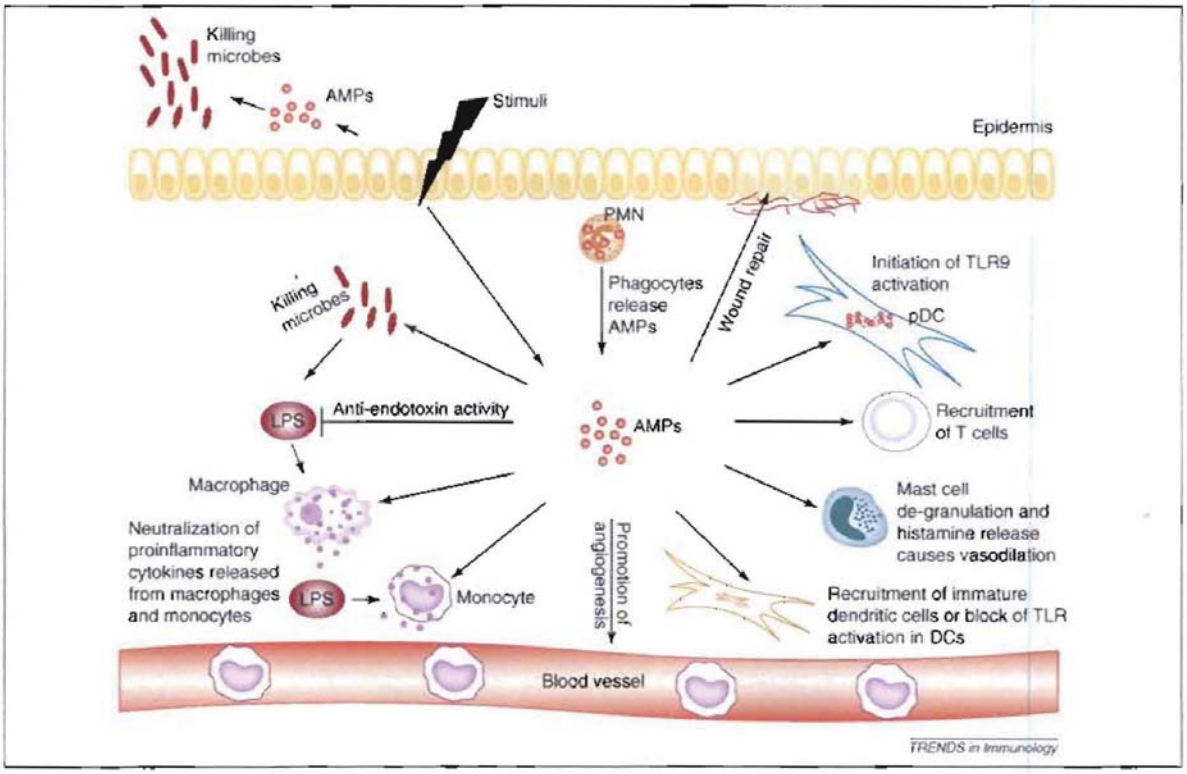

Figure 2.3 - A summary of AMP functions in the body (Lai & Gallo, 2009).

2.7 Hemoglobin

2.7.1 Functions of Hemoglobin

Simple organisms can receive sufficient oxygen for respiration via diffusion alone. As the size and complexity of organisms increase, this method is no longer sufficient to support life forms. As a result of evolution, there became a need for a proper circulatory system with an oxygen-carrying molecule (Maclean, 1978).

Hemoglobin is a protein component of red blood cells that reversibly binds oxygen and transports it from the lungs or gills of vertebrates to the body's tissues. Some invertebrates also use hemoglobin as an oxygen-carrying molecule. In invertebrates, hemoglobin may be found free in the haemolymph or within red blood cells of some species (Maclean, 1978). Plants, prokaryotes (Kosmachevskaya & Topunov, 2009) and fungi such as yeast (Maclean,

1978) also use hemoglobin and similar molecules to bind oxygen and regulate other molecules.

Although hemoglobin is a well studied protein, it was thought, up until the 1970s, that oxygen transport was its sole purpose. There is now evidence that whole hemoglobin, its subunits, and fragments of the protein have other significant bioactive properties, including antimicrobial activity.

2.7.2 Hemoglobin Structure

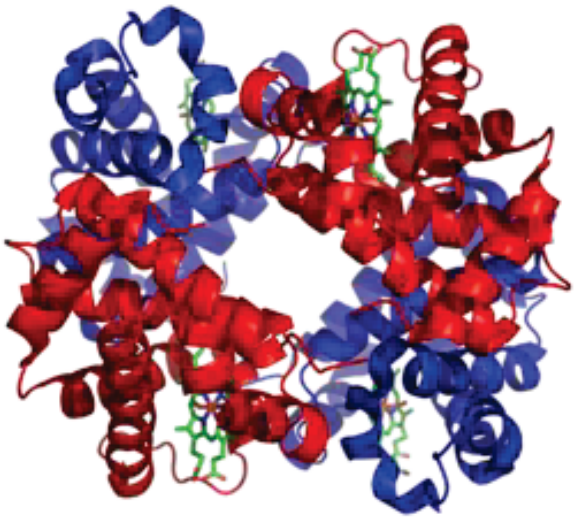

Figure 2.4 - Hemoglobin structure (RCSB Protein Data Bank, 2012).

Hemoglobin consists of four protein subunits, two alpha and two identical non-alpha subunits, which form a tetrahedral quaternary structure, as seen in Figure 2.4. The protein's structure consists largely of alpha helices, which are stabilised by hydrogen bonds. Short non-helical segments exist between helices (Bauer & Jung, 1975). Each subunit has an internal hydrophobic pocket that is tightly associated with a heme group-a ferrous ion that coordinates itself with four nitrogen atoms of four cyclically linked pyrrole groups. This is also known as a porphyrin ring (Maclean, 1978), and can been seen in Figure 2.5.

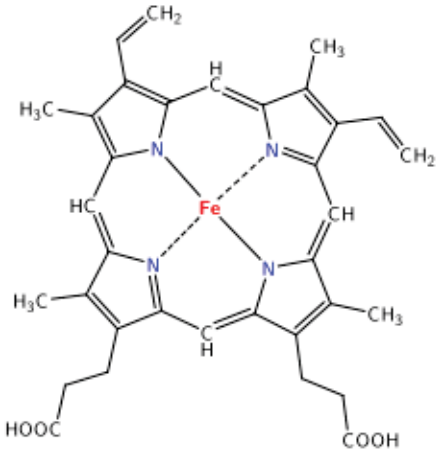

Figure 2.5 - Heme group (Maclean, 1978).

A heme group is linked to each globular subunit by non-covalent bonding of its ferrous ion to the imidazole ring of the F8 histidine. The last, unoccupied binding site to the iron ion is the site of oxygen binding. When oxygen becomes bound to deoxy-hemoglobin, the iron is temporarily oxidised from its ferrous state (Fe^{2+}) to its ferric state (Fe^{3+}) and moves into the plane of the porphyrin ring (Bauer & Jung, 1975).

Since each hemoglobin molecule has four heme groups, up to four oxygen atoms can be bound to the molecule at once (Maclean, 1978). Oxygen binding is cooperative; when one subunit binds oxygen, it causes conformational changes in the other binding sites, increasing the affinity of the other subunits for oxygen. This effect increases with each successive bound oxygen.

2.7.2.1 Developmental Variations of Hemoglobin

Different types of hemoglobin are present in the body in varying concentrations during an organism's development. Many organisms, such as sheep and humans, have embryonic, fetal and adult hemoglobins. These different hemoglobins possess properties essential for survival at each particular stage of development. For example, fetal hemoglobin has a greater affinity for oxygen than adult hemoglobin. This is necessary so that the fetus can obtain sufficient oxygen from the maternal blood supply (Berg et al., 2002).

As shown in Table 2.3, a total of six variations of the tetramer can exist during human life (Maclean, 1978). From two months into development, up until birth, embryonic hemoglobin is replaced by fetal hemoglobin. Then, after birth this is replaced by adult hemoglobin, of which type 'A' accounts for 97.5% of the total hemoglobin content (Maclean, 1978). Disease-causing variants also exist however. There is much variation in the number of hemoglobin types that exist within different species, and the concentration of these during development.

As mentioned earlier, hemoglobin consists of two alpha subunits, and two non-alpha subunits, which may be beta, sigma, gamma or epsilon subunits. It is thought that myoglobin was the template that the alpha subunit of hemoglobin arose from by gene duplication or translocation. Next, beta and gamma subunits emerged, followed by the sigma subunit, which evolved from the beta genome (Bauer & Jung, 1975).

Table 2.3 - Human hemoglobins and their subunits at varying developmental stages (Maclean, 1978).

Haemoglobins	*Symbols*	*Globin subunits*
Embryonic haemoglobins	HbE_1	$\alpha_2 \epsilon_2$ (Gower 2)
	HbE_2	$e_2 \zeta_2$ (Gower 1)
	HbE_3	$\zeta_2 \gamma_2$ (Portland)
Fetal haemoglobin	HbF*	$\alpha_2 \gamma_2$
Adult haemoglobins	HbA	$\alpha_2 \beta_2$
	HbA_2	$\alpha_2 \delta_2$

2.7.2.2 Hemoglobin Sequence Variations within a Species

Even within the same species natural variations (polymorphisms) of hemoglobin sequence occur. For example, literature often provides two slightly different sequences for the adult beta subunit of *Ovis aries*. This is because sheep have two allelic adult beta chains, versions A and B. These two versions differ by seven amino acids at six positions, as shown by Table 2.4. Cattle and goats also have more than one allele for the adult beta subunit of hemoglobin.

Table 2.4 - Sites of amino acid variations in ovine hemoglobin beta subunit, encoded by alleles A or B (UniProtKB, 2010).

Natural variations

Natural variant	49	1	N → S in allele A.
Natural variant	57	1	P → A in allele A.
Natural variant	74 – 75	2	MK → VQ in allele A.
Natural variant	119	1	N → S in allele A.
Natural variant	128	1	D → E in allele A.
Natural variant	143	1	K → R in allele A.

As a consequence of different beta subunit alleles, sheep have two adult forms of hemoglobin, Hb A and Hb B, for which an individual sheep may be homozygous or heterozygous (Huisman et al., 1965). It is known that Hb A has a higher affinity to oxygen than Hb B, and therefore sheep homozygous for Hb A may have a higher resistance to hypoxia (Bauer & Jung, 1975).

Interestingly, studies have shown that the B allele of the hemoglobin beta subunit in sheep is far more common than the A version. Wang et al. (1991) researched the allele frequencies of 11 domesticated sheep breeds and three genetic groups of wild sheep in the USA. It was found that the pooled frequency of allele B was high, at 0.71. Furthermore, it was discovered that the wild sheep only possess the B form, unlike the domestic populations, which all contained both allele types, with the exception of one breed. The group concluded that the presence of only B alleles in wild sheep, and the higher frequency of B alleles compared to A in domestic sheep indicates that allele A is newer and likely arose after the domestication of sheep.

Furthermore, sheep and goat that are homozygous or heterozygous for the HbA can switch to synthesising another hemoglobin type, hemoglobin C (HbC), if the animal is suffering anemia or hypoxia. This hemoglobin contains two alpha subunits, plus two beta allele C subunits. Sheep that are homozygous for HbB lack the genes to produce HbC. HbC is

normally a juvenile hemoglobin type, which gets replaced by adult hemoglobin after birth. Under conditions of erythropoietic stress, the synthesis of HbC is advantageous as it has a higher oxygen affinity than both regular adult forms (Benz et al., 1978).

2.7.2.3 Conservation of Hemoglobin Sequence between Species

Similarities in the hemoglobin amino acid sequence between species reflects how related they are on the evolutionary tree. Figure 2.6 shows the similarities and differences in the sequence of beta and alpha subunits between species. As a result of mutations that occurred during the course of evolution, the primary structure of the subunits varies slightly between species. However, there is little difference in the overall tertiary conformation of hemoglobin between species. This is because despite any differences in primary structure, hemoglobin must still maintain a conformation that allows fulfilment of its primary function - to reversibly bind oxygen (Bauer & Jung, 1975).

Beta subunits

```
Sheep (Ovis aries):allele A
1 mltaeekaav tgfwgkvkvd evgaealgrl lvvypwtqrf fehfgdlssa davmnnakvk

Sheep (Ovis aries):allele B
1 mltaeekaav tgfwgkvkvd evgaealgrl lvvypwtqrf fehfgdlsna davmnnpkvk

Human (Homo sapiens)
1 mvhltpeeks avtalwgkvn vdevggealg rllvvypwtq rffesfgdls tpdavmgnpk

Cow (Bos taurus)
1 mltaeekaav tafwgkvkvd evggealgrl lvvypwtqrf fesfgdlsta davmnnpkvk

House mouse (Mus musculus)
1 mvhltdaeka avsglwgkvn sdevggealg rllvvypwtq ryfdsfgdls sasaimgnak

Chicken (Gallus gallus)
1 mvhwtaeekq litglwgkvn vaecgaeala rllivypwtq rffasfgnls sptailgnpm



61 ahgkkvldsf sngvqhlddl kgtfaqlsel hcdklhvdpe nfrllgnvlv vvlarhhgse

61 ahgkkvldsf sngmkhlddl kgtfaqlsel hcdklhvdpe nfrllgnvlv vvlarhhgne

61 vkahgkkvlg afsdglahld nlkgtfatls elhcdklhvd penfrllgnv lvcvlahhfg

61 ahgkkvldsf sngmkhlddl kgtfaalsel hcdklhvdpe nfkllgnvlv vvlarnfgke

61 vkahgkkvit afneglnhld slkgtfasls elhcdklhvd penfrllgnm ivivlghhlg

61 vrahgkkvlt sfgdavknld nikntfsqls elhcdklhvd penfrllgdi liivlaahfs
```

121 ftpvlqaefq kvvagvanal ahryh

121 ftpvlqadfq kvvagvanal ahkyh

121 keftppvqaa yqkvvagvan alahkyh

121 ftpvlqadfq kvvagvanal ahryh

121 kdftpaaqaa fqkvmagvat alahkyh

121 kdftpecqaa wqklvrvvah alarkyh

Alpha subunits

Sheep (Ovis aries)
1 mvlsaadksn vkaawgkvgg nagaygaeal ermflsfptt ktyfphfdls hgsaqvkghg

Human (Homo sapiens)
1 mvlspadktn vkaawgkvga hageygaeal ermflsfptt ktyfphfdls hgsaqvkghg

Cow (Bos taurus)
1 mvlsaadkgn vkaawgkvgg haaeygaeal ermflsfptt ktyfphfdls hgsaqvkghg

House mouse (Mus musculus)
1 mvlsgedksn ikaawgkigg hgaeygaeal ermfasfptt ktyfphfdvs hgsaqvkghg

Chicken (Gallus gallus)
1 mvlsaadknn vkgiftkiag haeeygaetl ermfttyppt ktyfphfdls hgsaqikghg

61 ekvaaaltka vghlddlpgt lsdlsdlhah klrvdpvnfk llshsllvtl achlpndftp

61 kkvadaltna vahvddmpna lsalsdlhah klrvdpvnfk llshcllvtl aahlpaeftp

61 akvaaaltka vehlddlpga lselsdlhah klrvdpvnfk llshsllvtl ashlpsdftp

61 kkvadalana aghlddlpga lsalsdlhah klrvdpvnfk llshcllvtl ashhpadftp

61 kkvvaaliea anhiddiagt lsklsdlhah klrvdpvnfk llgqcflvvv aihhpaaltp

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121 avhasldkfl anvstvltsk yr
121 avhasldkfl asvstvltsk yr
121 avhasldkfl anvstvltsk yr
121 avhasldkfl asvstvltsk yr
121 evhasldkfl cavgtvltak yr
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Figure 2.6 – Comparision of hemoglobin alpha and beta chain sequences between species (NCBI, 2010).

2.7.3 History of Bioactive Peptides from Hemoglobin

In 1958 Hobson & Hirsh noted the first evidence of native hemoglobin possessing antimicrobial properties (Dubin et al., 2005; Hobson & Hirsch, 1958). They discovered that various hemoglobin species had an inhibitory effect on Gram-negative bacteria under certain conditions *in vitro*.

The first bioactive peptide from hemoglobin was discovered in 1971 by Schally et al. who isolated a peptide from pig hypothalamus, which possessed growth hormone-releasing ability. It was found that the peptide corresponded to residues 1-10 of the beta chain of hemoglobin (Liepke et al., 2003).

Since then, many biologically active peptides derived from hemoglobin have been isolated, including those with analgesic, antimicrobial, bradykinin-potentiating, opioid (Nedjar-Arroume et al., 2006), hemopoietic, coronaro-constrictory, hormone-releasing, immunomodulatory and antigonadotropic properties (Mak et al., 2000).

In 1986, Brantl et al. first reported that enzymatically (pepsin) treated bovine hemoglobin produced peptides with opioid-like properties (Daoud et al., 2005; Liepke et al., 2003). Opioid peptides from hemoglobin have now been classified as hemorphins, and they have also been found to naturally occur in the brain, plasma and cerebrospinal fluid (Nyberg et al., 1997).

The first discovery of an antimicrobial peptide from hemoglobin was a fairly recent event by Fogaca et al. in 1999. They identified a fragment in the gut of the tick *Boophilus microplus*, which corresponded to the sequence 33-61 of the alpha subunit of bovine hemoglobin and

had activity against Gram positive bacteria and fungi. It was proposed that the tick proteolytically degrades bovine hemoglobin to produce antimicrobial peptides, protecting the tick itself from microbial invasion.

Since these milestones, the literature has reported the discovery of many more antimicrobial peptides derived from both the alpha and beta subunits of hemoglobin, mainly in humans and cattle. Mak et al. (2000) have now described these antimicrobial peptides derived from heme-containing proteins as hemocidins.

2.7.4 Formation of Bioactive Hemoglobin-derived Peptides

2.7.4.1 *In vivo*

There has been a lot of discussion as to how biologically active hemoglobin-derived peptides are formed in the body. Originally it was thought that these peptides or fragments were a result of non-specific proteolytic degradation of hemoglobin once it had fulfilled its primary physiological function (Dubin et al., 2005; Karelin et al., 1998). However, studies suggest that peptide formation from hemoglobin may be a complex process involving a specific and regulated system of tissue specific enzymes and substrates (Karelin et al., 1998; Mak, 2008).

Hemoglobin peptides are currently the largest group of protein fragments found in tissue extracts, making up 30-90% of sequenced protein fragments (Karelin et al., 1998). Their concentration in tissues is higher than in blood plasma or cerebrospinal fluid, suggesting their action exerted here (Karelin et al., 1998).

A range of bioactive hemoglobin peptides have been found in many different tissues and organs of species such as humans, pigs, cows and squirrels (see Table 2.5).

Table 2.5 – Origin and bioactive functions of hemoglobin peptides (Karelin et al., 1995).

Location in Hemoglobin	Source	Biological Activity	References
α-1–8	bovine bone marrow	hemopoietic in vivo	9
α-1–21	bovine hypothalamus	coronaro-constrictory in vitro	1
α-33–38	pig bone marrow	nociceptive in vivo	5
		immunomodulatory in vitro	5
α-33–46	pig brain	ACTH-releasing in vitro	13
α-110–124	bovine hypothalamus	coronaro-constrictory in vitro	1
α-137–139	bovine pituitary gland	antigonadotropic in vivo	12
α-137–141	bovine brain	analgesic in vivo	15
	human tumor cell culture	analgesic in vivo	18
	squirrel brain	antihibernatic in vivo	17
		ion current regulation in vitro	14
α-140–141	bovine brain	analgesic in vivo	16
β-31–38	bovine brain	convulsion in vivo	9
β-31–39	human pituitary gland	opioid-like in vivo	6
		opioid-like in vitro	6
		ACE activity in vivo	11
β-31–40	pig hypothalamus	not known	3
	human liquor	opioid-like in vivo	7
		opioid-like in vitro	7
	bovine hypothalamus	coronaro-constrictory in vitro	1
β-32–37	pig bone marrow	nociceptive in vivo	5
		immunomodulatory in vitro	5
β-32–38	bovine hypothalamus	opioid-like in vivo	4
		opioid-like in vitro	4
		coronaro-constrictory in vitro	1
β-32–39	bovine hypothalamus	coronaro-constrictory in vitro	1
β-32–40	pig hypothalamus	not known	3
β-33–37	bovine hypothalamus	coronaro-constrictory in vitro	1
β-33–38	bovine hypothalamus	coronaro-constrictory in vitro	1
β-133–146	bovine hypothalamus	coronaro-constrictory in vitro	1

Since hemoglobin is found in high concentrations in erythrocytes, it might be assumed that hemoglobin peptides found in tissues originate from erythrocytes. However, it has been found that most hemoglobin fragments found in tissues differ from those formed inside or secreted by erythrocytes. For example, it has been shown that only a few hemoglobin peptides present in the rat brain are in common with those inside erythrocytes. Also, 'the concentration of hemoglobin fragments in rat brain is approximately 10 times higher than the concentration of corresponding intraerythrocyte peptides in the blood' (Ivanov et al., 2005). Therefore, Ivanov et al. (2005) state that it is not possible for intraerythrocyte peptides to be precursors for peptides found in tissues. Moreover, there is controversy as to

whether peptides that are produced in erythrocytes have antimicrobial activity. Liepke et al. (2003) discovered an antimicrobial fragment from the hemoglobin beta subunit in lysed human erythrocytes. However, Fogaca et al. (1999) did not detect any activity from lysed bovine erythrocytes. Liepke et al. (2003) proposed that this could be due to the different species of erythrocytes, the different test conditions or the bacterial strains used for testing.

Furthermore, hemoglobin fragments differ greatly between different tissues. Peptide/fragment concentration and sequence were found to be highly tissue specific (Karelin et al., 1998) and it has been suggested that hemoglobin maybe degraded in tissues by tissue specific proteinases resulting in unique peptides in each tissue. In the case of hemocidins found in menstrual blood, it has been proposed that the acidic conditions of the vagina facilitate hemoglobin release from red blood cells and partial denaturation (Mak et al., 2004). Proteases specific to the vagina then proceed to digest hemoglobin into antimicrobial fragments.

To date, AMPs from hemoglobin have been isolated *in vivo* from sources including: the hemolymph and gut contents of the cattle tick (Fogaca et al., 1999), human menstrual blood (Mak et al., 2004) and placental tissue (Liepke et al., 2003), and in the skin and gill epithelium of catfish challenged with the parasite, ichthyophthirius multifiliis (Ullal et al., 2008). There needs to be further research on this topic to clarify mechanisms of proteolytic hemoglobin degradation, the conditions required, and the specific proteases involved.

2.7.4.2 *In vitro*

In vitro, bioactive hemoglobin-derived peptides can be created by partial chemical or enzymatic digestion of hemoglobin (Daoud et al., 2005; Mak et al., 2000). This is true for other heme-containing proteins also, such as myoglobin and cytochrome c (Mak et al., 2000).

Froidevaux et al. (2001) was the first to report an AMP isolated *in vitro*, from a peptic bovine hemoglobin hydrolysate. The peptide corresponded to residues 1-23 of the alpha subunit, and presented weak bactericidal properties against *Micrococcus luteus* A270.

Since then, numerous reports on the use of pepsin in the production of antimicrobial peptides from bovine hemoglobin have been published. The use of RP-HPLC appears to be the chosen method for purifying hydrolysates into peptide fractions.

Chemicals and enzymes other then pepsin can be used in the digestion of hemoglobin to obtain antimicrobial peptides. In an attempt to determine antimicrobial domains of hemoglobin, Mak et al. (2000) and Parish et al. (2001) used cyanogen bromide (CNBr) to cleave alpha and beta subunits into fragments, and then determine the antimicrobial activities of the generated peptides. Mak et al. (2000) also digested with trypsin in order to obtain shorter fragments, which may coincide with the larger produced CNBr fragments, providing further information on the location of these critical sequences. However, the fragments from trypsin digestion had limited activity, suggesting that over-fragmentation disrupted the sequences essential for antimicrobial activity.

Recently, Catiau et al. (2011b) used the same concept to find the minimum antimicrobial sequence of the bovine alpha chain. Bovine hemoglobin was digested with porcine pepsin and the activity of the fragments against various Gram positive and Gram negative bacteria were determined. Fragment alpha 137-141 possessed the strongest activity, and fragments of this decreasing by one amino acid at a time were synthetically produced i.e. alpha 138-141, 139-141 and 140-141. Peptide alpha 139-141 (amino acid sequence KYR) had the strongest activity, and was stated to be the minimum sequence for antimicrobial activity of the alpha chain. The group later utilised the same concept on the beta chain and found that the minimum sequence for antimicrobial activity here is beta 143-145 (RYH), which also had the lowest MIC (Catiau et al., 2011a). It was concluded that peptides with strong antimicrobial activity contained the amino acid sequence ‘YR’ (tyrosine, arginine), but this dipeptide itself was inactive.

2.7.4.2.1 Pepsin Digestion of Hemoglobin

Choisnard et al. (2002) studied the kinetics of antimicrobial peptide appearance in a bovine hemoglobin peptic hydrolysate over time. Their primary focus was on identifying the hydrolysis conditions necessary to produce the maximal concentration of peptide alpha 1-

23, the first antimicrobial hemoglobin fragment to be isolated from a hemoglobin hydrolysate.

A comparison of kinetics was made between two sets of hydrolysis conditions. In the first, hemoglobin was present in a sodium acetate buffer in its native state. In the second condition, the peptic digest was carried out in the presence of urea, allowing for denatured hemoglobin as the substrate.

It was found that under the urea conditions, peptide alpha 1-23 was produced at twice the concentration compared to those conditions lacking urea. Furthermore, in the presence of urea, this peptide, like other peptides, appeared much earlier in the digestion. Observation of the RP-HPLC profiles of the two experimental conditions clearly shows the rapid degradation of hemoglobin under the urea conditions. This is due to the fact that hemoglobin denaturation makes peptide bonds more accessible to pepsin. Hemoglobin can also be denatured by utilising low pH conditions (pH 2), rather than urea (Zhao et al., 1996).

Furthermore, Adje et al. (2011) showed that the use of various alcohols can modify hemoglobin structure to a state that is neither its native state nor fully denatured. Peptic hydrolysis under these conditions allows for the formation of new antimicrobial intermediate peptides, which are not present in the prior conditions discussed.

2.7.5 Antimicrobial Activity of Hemoglobin, its Subunits and Peptides

Since the first reports of antibacterial activity of extracts from bovine erythrocytes by Hobson & Hirsch (1958), other research groups have analysed the activity of whole hemoglobin, its subunits and peptides.

Mak et al. (2000) reported that neither intact human hemoglobin nor whale myoglobin had antimicrobial activity against a range of organisms - *Escherichia coli*, *Salmonella*, *Pseudomonas aeruginosa, Klebsiella oxytoca, Enterococcus faecalis, Staphylococcus aureus* and *Candida albicans*. However, when these proteins were deprived of heme and partially unfolded they were antimicrobially active against all tested organisms (Mak et al., 2000).

On the other hand, a year later Parish et al. (2001) tested the activity of intact alligator, human, horse, and snake hemoglobins. All hemoglobins had strong MICs of 2 to 25ug/ml against *E.coli*, as shown in Table 2.6. No species of hemoglobin was active against *S.aureus*, but snake hemoglobin was particularly active against all the other target organisms (Parish et al., 2001). Discrepancies between these two research groups maybe a result of the different antimicrobial assays used.

Like Mak et al. (2000), Parish et al. (2001) discovered that when hemoglobin is unfolded into its alpha and beta subunits, the antimicrobial activities increase dramatically. Furthermore, although the subunits had good activity with heme-intact, the activity was greater without heme in most cases. It was proposed that heme is not necessary for antimicrobial activity, and that the separation of subunits exposes antimicrobial regions that aren't accessible in the intact protein.

Table 2.6 - Comparison of MIC values between intact hemoglobin, alpha and beta subunits with or without heme attached, and hemoglobin peptides (Parish et al., 2001).

Entry	Test substance	Target organisms			
		Escherichia coli Gram negative	*Streptococcus faecalis* Gram positive	*Staphylococcus aureus* Gram positive	*Candida albicans* Fungus
Intact hemoglobins:					
1	Alligator Hb	**25**[a]	ND[b]	ND	20,000
2	Human Hb	**2**	1500	20,000	**50**
3	Horse Hb	**2**	20,000	20,000	250
4	Snake Hb	**10**	**15**	20,000	**100**
Human hemoglobin fragments:					
α peptides					
5	α + heme	**1**	**2**	ND	**15**
6	α-heme	**2**	**3**	15	7
7	I α (1–32)	(> 100)	(> 100)	(> 100)	(> 100)
8	II α (33–76)	**100**	100	(> 350)	**70**
9	III α (1–76)	(> 470)	300	(> 470)	(> 470)
10	IV α (77–141)	(> 100)	(> 100)	(> 100)	**30**
β peptides					
11	β + heme	**3**	**15**	ND	10,000
12	β-heme	**4**	**20**	200	300
13	V β (56–146)	**5**	**8**	**60**	**25**
14	VI β (1–55)	**100**	**15**	**60**	**25**
15	β (116–146)[c]	**2**	2500	**50**	**10**
16	β (56–72)[d]	**20**	10,000	ND	1500

[a]The values are given in μg/mL and correspond to the minimum inhibitory concentration (MIC) obtained as the intercept on the horizontal axis from results similar to those shown in Figure 1. MIC values less than or equal to 100 μg/mL are shown in bold. Each assay was performed in triplicate several times. The results in the Table are derived from a single determination performed in triplicate. Values in parentheses indicate that the sample had no activity at the indicated highest concentration that was tested.
[b]Not determined.
[c]β (116–146) sequence: HHFGKEFTPPVQAAYQKVVAGVANALAHKYH.
[d]β (56–72) sequence: GNPKVKAHGKKVLGAFS.

In many cases, peptides generated from hemoglobin *in vitro* or those found *in vivo* are reported to possess greater antimicrobial activity than intact hemoglobin or its subunits. For example, a hemoglobin fragment from cyanogen bromide treatment (human HbB(56-146)) was found to have a potent MIC of 1.2uM towards *E.coli* (Mak et al., 2000). However, the relationship between peptide structure and the degree of antimicrobial activity appears to be complex.

Nedjar-Arroume et al. (2008) generated numerous AMPs from peptic digestion of bovine hemoglobin. They noted that the peptides could be divided into two groups based on their properties. The first was peptides with typical AMP characteristics i.e. they had a net positive charge, a moderate number of hydrophobic residues and were mostly alpha helical in structure. The second group were also positively charged but were small (<15 amino acids), lacked secondary structure, and had no or few hydrophobic residues. Interestingly, it was the second group of peptides that had by far the most potency towards microorganisms, at 1 to 15uM.

2.7.6 Hemocidin Mechanism of Action

It is thought that alpha helical and random coiled hemocidins utilise two different membrane disruption mechanisms (Nedjar-Arroume et al., 2008). A lot more information is known on the mechanism of alpha helical hemocidins.

A likely mechanism for antimicrobial activity of alpha helical hemocidins has been reported by Mak et al. (2000). This was based on their study of fragment 56-131 from horse apomyoglobin (heme-free myoglobin), produced by cyanogen bromide cleavage. They discovered that this peptide has a disordered structure in aqueous solutions. When the peptide comes into contact with the membrane phospholipid bilayer, non-specific electrostatic interactions occur and the peptide folds into an alpha helical form, giving contact with the membrane. Aggregation of peptides then occurs on the membrane surface, forming carpet-like structures. These induce curvature, destabilising the membrane in a detergent-like manner, leading to cell disruption. The action of this peptide has been described as a carpet-like mechanism; the peptide causes membrane disruption due to the

formation of 'carpet-like' structures on the membrane surface, without channel-forming. Furthermore, it was found that the bilayer lifetime is dependent on peptide concentration at the membrane surface.

In terms of random coiled hemocidins, Nedjar-Arroume et al. (2008) state that 'Perhaps these peptides act by a mechanism closer to the antibacterial activity of parabens than the activity of amphipathic peptides by involving their terminal tyrosyl group.'

2.7.7 Factors that Affect Hemocidin Activity

The activity of AMPs is influenced by many factors. These commonly include: pH, salt concentration, metal ion concentration, the presence of serum proteins and temperature. Under physiological conditions, many of these factors are antagonistic to the antimicrobial activity of peptides. Due to this, most AMPs are only active under conditions *in vivo* of low salt and serum concentration, such as in the vacuoles of phagocytes, and on skin surfaces and mucosal epithelium (Jones et al., 2005). Therefore, although a peptide may exert strong antimicrobial activity *in vitro*, it may not *in vivo*, depending on how sensitive the peptide is to the above factors.

An article by Mak et al. (2007) discusses the effect of pH, salt concentration, and divalent ion concentration on the activity of an antimicrobial peptide from the beta subunit of human hemoglobin. The peptide, human HbB115-146 (AHHFGKEFTPPVQAAYQKVVAGVANALAHKYH), was found to be one of the most active antimicrobial peptides in the menstrual discharge from the vagina; it is active against Gram positive and Gram negative bacteria, particularly *E.coli*, and mildly active against *C.albicans* (Mak et al., 2004).

Under various physicochemical conditions, bacterial membrane permeability tests were carried out using beta-galactosidase transformed *E.coli* cells. The results can be seen in Figure 2.7.

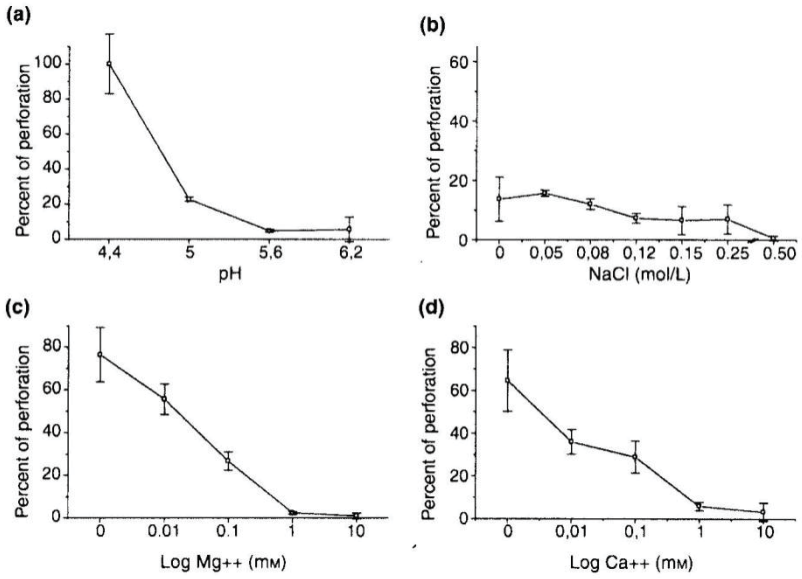


Figure 2.7 - Perforation of *E.coli* membrane by human HbB115-146 under varying pH, salt concentration, and divalent cation concentration. The final peptide concentration was 16umol/L for the pH-dependent experiment, and 16.7umol/L for salt-resistance and divalent cation experiments (Mak et al., 2007).

The activity of the peptide was shown to be highly affected by pH: 'The change of pH from 4.4 to 5.0 reduced activity of the peptide by 80%' (Mak et al., 2007). Very little perforation activity occurred at a pH greater than 5.0.

The effects of the diavalent cations, Mg^{2+} and Ca^{2+}, on cell perforation potential were tested. As expected there was a dramatic decrease in the perforation percentage of cells as the concentration of these cations increased: 'about 95% inhibition was observed with 1mM Mg^{2+} or Ca^{2+}' (Mak et al., 2007). This decrease in peptide activity is due to its competition with the diavalent cations for binding sites on the negatively charged membrane. It also suggests that the antimicrobial activity of this peptide would be inhibited in most locations in the body, as the physiological concentration of these cations is higher than this i.e. 1.8-2.5mM for Mg^{2+} in serum.

Atypical of antimicrobial peptides is the fact that the activity of HbB115-146 was barely affected by the concentration of NaCl, up to 0.25mol/L. Most cationic antimicrobial peptides show a significant decrease in activity at even physiological salt concentration (0.15mol/L). The reason for this unique behaviour by HbB115-146 is not understood.

In summary, HbB115-146 exhibits antimicrobial activity in low pH conditions existing in the vagina. Its activity is affected by diavalent cations, and surprisingly, it is not very sensitive to salt concentration.

Mak et al. (2000) also carried out the same tests on the antimicrobial peptide, 56-131, from horse apomyoglobin. A significant decrease in membrane perforation due to an increasing divalent cation concentration was observed, as with peptide HbB(115-146). This peptide however, had optimum activity at a higher pH of 6-7. It was also salt sensitive near physiological salt concentrations, unlike HbB(115-146). Figure 2.8 below shows evidence of this.

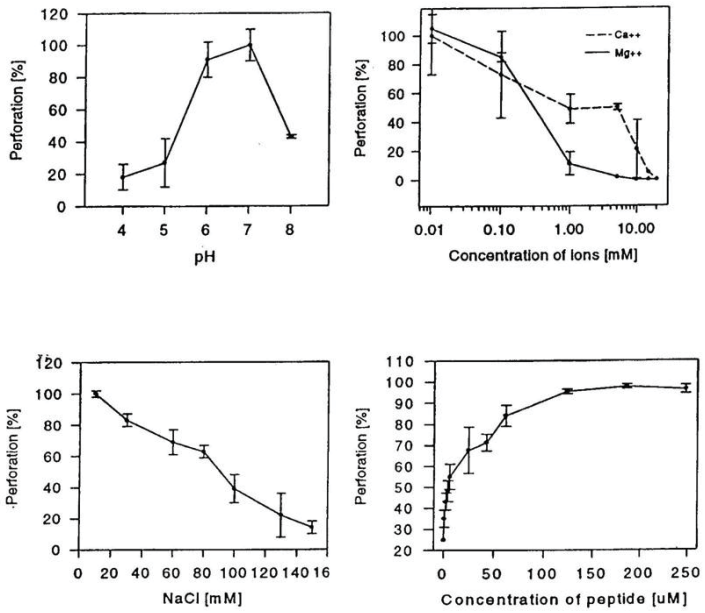


Figure 2.8 – Perforation of *E.coli* membrane by horse myoglobin peptide 56-131 under varying pH, salt concentration, divalent cation concentration, and peptide concentration. The final peptide concentration was 1.5umol/L (Mak et al., 2000).

Another factor that can influence the antimicrobial activity of a peptide is the growth phase of the microorganism. Many antimicrobial assays state the use of cells in their mid-logarithmic phase. This is because AMPs may be more effective against metabolically active or growing microorganisms (Lehrer et al., 1991). However, a discussion of this topic by Yang & Oppenheim (2004) suggests that this may be dependent on the peptide. For example, some peptides have equal killing activity against cells in either their stationary or log phase, others kill cells in the log phase quicker than in the stationary phase, while some have almost no activity against stationary phase organisms.

2.8 Applications of AMPs

2.8.1 AMPs as Therapeutics

The increasing development of antibiotic resistant microorganisms and the lack of new effective antibiotics have provided the driving force for the investigation into AMPs as potential anti-infectives.

Microorganisms are becoming resistant to antibiotics at a rate faster than new ones can be designed and produced (Giuliani et al., 2007) and the majority of new antibiotics produced by pharmaceutical companies are simply modifications of existing antibiotics rather than new novel classes (Gordon et al., 2005). Hence there is a need for new and novel antimicrobials to replace or supplement existing antibiotics.

2.8.1.1 Advantages and Disadvantages

Although AMPs have many desirable features for therapeutic drug development they also have potential disadvantages, which need to be further investigated. For this reason, currently there have been no reports on the commercialisation of any therapeutic AMP agents (Gordon et al., 2005), although many are in preclinical and clinical trials.
Table 2.7 lists some of the primary advantages AMPs have for drug development over existing antibiotics, including fast acting broad spectrum activity, and lack of resistance development by microbes. Other benefits not included in Table 2.7 are the ability for synergy with other AMPs or existing antibiotics, and endotoxin binding (Giuliani et al., 2007).

However, there are many unknowns as to how AMP drugs will interact with a mammalian body. Peptides that provide promising results *in vitro* studies often lack effectiveness when trialled in animal models. This is due to the naturally occurring antagonistic conditions of the body, such as, ionic and pH changes, the presence of serum proteins, occurrence of proteolysis, poor tissue penetration and immune clearance from prior sensitisation (Gordon et al., 2005). Also, AMPs may have unwanted side effects, including cytotoxicity and hemolysis. Furthermore, AMP drug design is difficult due to the complex interactions of

peptides with membranes and each other, and the lack of correlation between structural characteristics and activity (Koczulla & Bals, 2003).

Table 2.7 – Advantages and disadvantages of AMPs as therapeutics (Gordon et al., 2005).

Advantages
Broad-spectrum activity (antibacterial, antiviral, antifungal)
Rapid onset of killing
Cidal activity
Potentially low levels of induced resistance
Concomitant broad anti-inflammatory activities
Disadvantages
Discovery costs of synthesis and screening
Patent exclusivity for economic viability
Systemic and local toxicity
Reduced activity based on salt, serum, and pH sensitivity
Susceptibility to proteolysis
Pharmacokinetic (PK) and pharmacodynamic (PD) issues
Sensitization and allergy after repeated application
Natural resistance (e.g., *Serratia marcescens*)
Confounding biological functions (e.g., angiogenesis)
High manufacturing costs

Another issue hindering the commercialisation of AMP drugs is their high production cost. Currently, the production cost of synthetic peptides is 5 to 20 times as high as that of conventional antibiotics (Giuliani et al., 2007). However, it is said that the costs of peptide synthesis is decreasing, due to three factors:

1. The use of more cost effective production methods.

2. Selection of peptide candidates that are shorter, do not contain complex folds such as disulphide bonds, and are required in low concentrations to be effective *in vivo*.

3. Competition between recombinant production companies and synthetic peptide manufacturers (Falla & Zhang, 2004).

Although AMP production is currently reserved for high value clinical products, the lowering of production costs means that these peptides may in the future be found in non-clinical products such as in medical devices (Falla & Zhang, 2004) and in preservatives. The *in vivo* conditions discussed along with high production costs are the primary causes for the current lack of commercialisation.

2.8.1.2 Uses of AMPs as Therapeutic Drugs

Both the antimicrobial and immune mediating properties of AMPs can potentially be exploited in drug development. The broad categories of potential AMP uses include:

1. Single anti-infective agents.
2. In combination with antibiotics or antivirals to produce additive or synergistic effects.
3. Immunostimulatory agents to enhance innate immunity.
4. Endotoxin-neutralising agents to reduce or prevent sepsis (Gordon et al., 2005).

The use of AMPs as topical agents looks to be the most promising method of drug administration so far. This is because topical administration is safe in comparison to systemic administration, especially when drug toxicology is unknown (Giuliani et al., 2007). Furthermore, as mentioned earlier, antagonistic factors, such as proteases, ions and salt concentration are present in much lower concentrations on the skin, resulting in greater effectiveness of AMPs. Also, AMPs are naturally involved in local responses to infection in the body (Hancock & Lehrer, 1998). Hence, the first clinical trials of potential AMP therapeutics have been for topical infections.

Table 2.8 provides a summary of the commercial development of AMPs, including the product, company, outcomes of trials and future trials.

The first AMP to undergo commercial development was a cationic 22 residue peptide called, Pexiganan. It is an analogue of magainin 2 from the skin of an African clawed frog, *Xenopus laevis*. Genaera incorporated Pexiganan into a cream called Locilex, designed to treat polymicrobic diabetic foot ulcers (Falla & Zhang, 2004; Giuliani et al., 2007; Gordon et al., 2005). Despite its broad spectrum activity and resistance to mutant development, Locilex failed to gain FDA approval as it was considered no more effective than current antibiotic treatments, such as Ofloxacin, in phase III clinical trials (Gordon et al., 2005; Koczulla & Bals, 2003).

Canadian company, Migenix, is most advanced in their development of two indolicidin analogues, MX-226/Omiganan and MX-594AN (Giuliani et al., 2007). The first, MX-226, was

designed as a topical gel treatment for the treatment of catheter-related bloodstream infections, but failed to achieve this in the phase III clinical trial (Gordon et al., 2005). However, in the same study, MX-226 proved to significantly reduce catheter colonisation and reduce catheter-related local infections (Gordon et al., 2005). A confirmatory phase III trial is in the pipeline (Giuliani et al., 2007).

The second peptide, MX-594AN, is a topical antimicrobial for the treatment of acne vulgaris, directed at *Propionibacterium acnes.* In a phase IIb trial, inflammatory, non-inflammatory and total lesions were reduced significantly compared to the control (Gordon et al., 2005). A phase III trial is planned.

Other targets for clinical AMP treatments include eczema, cystic fibrosis, burn wounds, oral hygiene, STDs and infections frequent in HIV patients (Falla & Zhang, 2004).

Table 2.8 – Commercial development of AMPs (Giuliani et al., 2007).

	Product	Company	Trials outcome and applications	Planned trials
Available on the market	**Polymixin B-Colistin-Colomycin (prodrug)**	RX Generic drugs	Indicated for G- skin infections	none
	Daptomycin (Cubicin ®)	Cubist Pharmaceuticals	Indicated for G+ skin infections	not announced
Late clinical development (Phase III and above)	**Pexiganan**	Genaera PlymouthMeeting	Failed FDA approval: no advantage over convent. antibiotics.	none
	Iseganan	Intrabiotics Pharmaceuticals	Failed two Phase III: stomatitis and pneumonia.	Phase IIa trial as Rx for CF patients
	Omiganan	Migenix	Failed Phase III trial as Rx for catheter related bloodstream infections.	Repeat Phase III trial
Early clinical development (Phase I, II, pre-clinical)	**LTX Serie**	Litix Biopharma	Several G+, G- and fungal infections. Preclinical.	not announced
	SB006	SpiderBiotech	G- infections. Under preclinical trials.	not announced
	Product-I serie	Inimex	Several hospital bacterial infections.	not announced
	MX594AN	Migenix	Phase IIb showed efficacy as topical Rx for acne.	Phase III trial
	Plectasin	Novozyme	G- infections. Under preclinical trials.	not announced
	P113/P113D	Demegen/Pacgen	Phase IIb showed efficacy for oral candidasis.	Inhalation RX for CF patients under consideration
	hLF1-11	AM-Pharma	Positive Phase I.Under Phase II for severe fungal and bacterial infection.	not announced
	XMP.629	Xoma	Failed Phase II clinical trial as topical Rx for acne.	not announced
	Neuprex	Xoma	Failed Phase III as Rx in pedriatic meningococcemia.	Planned Phase I/II in pediatric indications
	Mersacidin	Novacta Biosystems	Systemic anti-MRSA and other G+ pathogens. Under preclinical trials.	not announced
	PTX serie	PepTx	G- infections and LPS neutralization (antiseptic). Under preclinical trials.	not announced
	HB Serie	Helix-Biomedix	Preclinical trials showed efficacy for cystic fibrosis.	not announced

2.8.2 AMPs as Food Preservatives

Nisin and pediocin PA-1 (a bacteriocin from another class with activity directed at *Listeria monocytogenes*), are the only examples of peptides used as food preservatives on a large scale (Cotter et al., 2005).

As mentioned earlier nisin is a bacteriocin produced by food grade *Lactococcus lactis* subsp. *lactis* during fermentation (Delves-Broughton, 2005), which inhibits Gram positive bacteria. In 1988, nisin gained US Food and Drug administration approval for use as a preservative in pasteurised, processed cheese spreads (Cotter et al., 2005). Now the peptide has approval for use in over 50 countries and is used in New Zealand to inhibit pathogenic and spoilage organisms in: cream products, crumpets, pikelets, cheese products, desserts, and sauces (Delves-Broughton, 2005). Although the peptide targets Gram positive bacteria, Gram negative bacteria are susceptible when membrane integrity is diminished (Delves-Broughton, 2005). This occurs during processes such as heat treatment, osmotic shock, freezing and exposure to chelating agents (Delves-Broughton, 2005). Advantages of nisin as a preservative, aside from food safety and prolonged shelf-life, are that the peptide is heat stable (Cotter et al., 2005), non-toxic, not in use clinically, digested quickly and bacteria do not develop resistance towards it (Delves-Broughton, 2005).

2.9 Conclusions

AMPs are such a diverse group of molecules that they can be classified according to many structural features. Based on their 3D structure and composition they may be linear with an alpha helical structure, beta sheets with disulphide bridges, and have a predominance of specific amino acids, or loop structures. It appears that the majority are cationic and amphipathic, with at least half the residues being hydrophobic.

There are two broad categories of AMPs. The first, for which there is much literature available are the classical AMPs. These are encoded by specific genes in the organism's genome, and include well characterised peptides from eukaryotes (defensins and cathelicidins) and those from bacteria e.g. bacteriocins. The second are those that arise due to proteolytic digestion of already functional proteins and substrates include: serum albumin, gluten, cytochrome c, hemoglobin, casein and lactoferrin. As well as formation of AMPs, numerous bioactive peptides are also generated in the same way from these proteins. Their various biological effects include: analgesic, bradykinin-potentiating, opioid, hemopoietic, coronaro-constrictory, hormone-releasing, immunomodulatory and antigonadotropic properties.

Mammalian AMPs may exert antimicrobial action in two ways: by direct interaction with microbial membranes and intracellular components, or by influencing the activities of effector cells in the immune system. The peptides kill microbes by mechanisms that destabilise membranes or create trans-membrane pores, which may or may not allow the peptides to act intracellularly also.

AMPs act as chemoattractants for specific receptors on host defence cells resulting in the recruitment of neutrophils, monocytes and macrophages to the infection site, in the innate immune system, and immature dendritic cells and T cells in the adaptive immune system. The peptides also indirectly recruit effector cells by inducing chemokine and cytokine expression. AMPs have numerous other roles in the body, including: wound healing, regulation of the complement system, enhancing phagocytosis and antisepsis functions.

It was found that hemoglobin primary structure varies with stages of organism development, within a species due to polymorphisms, and of course between species. However, its

tetrahedral quaternary structure has remained highly conserved over the course of evolution, as it must maintain its primary function of reversibly binding oxygen.

Although it was discovered in 1958 that whole hemoglobin has antimicrobial properties, the first report of a hemoglobin peptide having antimicrobial activity was only 13 years ago. It has been proposed that, *in vivo*, hemoglobin peptides are generated by proteolytic digestion by tissue specific proteases, resulting in a set of peptides unique to that tissue. *In vitro*, peptides are generated by partial enzymatic or chemical fragmentation.

There is conflicting evidence as to the length of hemoglobin peptides that can exert antimicrobial action. One research group found short peptides to have the strongest MICs, while another stated that peptides with less than 50 residues had diminished or completely abolished activity. Also, hemocidins with an alpha helical structure were proposed to act by a 'carpet-like' mechanism, while the mechanism for random coiled peptides was unknown.

Like classical AMPs, the antimicrobial activity of hemocidins was largely reduced in the presence of divalent cations, Mg^{2+} and Ca^{2+}. Typical of antimicrobial peptides, peptide 56-131 of horse apomyoglobin was highly sensitive to salt, whereas, unusually, HbB 115-146 activity was only slightly affected by physiological salt concentrations. Both peptides had very different optimal pH for antimicrobial activity, HbB 115-146, pH 4.4-5, and horse apomyoglobin 56-131, pH 6-7.

Lastly, it was found that AMPs have many desirable traits for use as therapeutic drugs and biopreservatives; they are broad spectrum, fast acting, resistance development is difficult, have synergistic effects with other molecules, and can bind endotoxins. However, peptides may lack effectiveness *in vivo* due to antagonistic factors or be toxic to the body. Because of this, they may be limited to topical use. These issues, as well as high production costs have meant that no therapeutic AMP products have been commercialised so far despite the many AMPs in clinical trials. Nisin however, has been used for years as a food preservative.

Chapter 3

Materials and Methods

3.1 Materials

- **Fresh Ovine Blood**

Fresh ovine blood was collected from the neck of Halal slaughtered sheep at the Feilding site of Ovation New Zealand Limited.

- **Bovine Hemoglobin**

Powdered hemoglobin from bovine blood was sourced from Sigma-Aldrich, St. Louis, MO, USA.

- **Porcine Pepsin**

Pepsin from porcine stomach mucosa (pepsin A; EC 3.4.23.1) was sourced from Sigma-Aldrich, St. Louis, MO, USA.

- **SDS-PAGE Protein Standard Marker**

For tricine SDS-PAGE, Bio-Rad (Hercules, CA, USA) broad range SDS-PAGE molecular weight standards were used.

Protein:	Molecular weight (Da):
Myosin	200, 000
Beta-galactosidase	116, 250
Phosphorylase B	97, 400
Bovine serum albumin	66, 200
Ovalbumin	45, 000
Carbonic anhydrase	31, 000
Soybean trypsin inhibitor	21, 500
Lysozyme	14, 400
Aprotinin	6, 500

- **Gel Filtration Media**

Bio-Rad (Hercules, CA, USA) Bio-Gel P-10 gel was used for desalting. The bead characteristics were: medium polyacrylamide beads for size exclusion chromatography, 90-180 µm wet bead size, 1,500-20,000 MW fractionation range.

- **Synthetic Peptides**

A lyophilised synthetic peptide corresponding to residues 129 to 145 of the beta chain of ovine hemoglobin (beta-B(129-145)) was used as an RP-HPLC external standard. Along with this peptide, three other peptides from various regions of the ovine hemoglobin sequence were synthesised for antimicrobial testing by Peptide 2.0 (Chantilly, VA, USA).

Origin:	Sequence:	Molecular weight:	Purity:
beta-B(129-145)	-FQKVVAGVANALAHKYH-	1853.1g/mol	99.50%
beta-B(140-145)	-LAHKYH-	767.9g/mol	99.23%
beta-A(140-145)	-LAHRYH-	795.9g/mol	99.94%
alpha(96-107)	-PVNFKLLSHSLL-	1367.7g/mol	93.96%

- **Microorganisms**

Three test microorganisms were used in the antimicrobial assays: Gram negative bacterium, *Escherichia coli 0111*; Gram positive bacterium, *Staphylococcus aureus NCTC 4163*; a fungus, *Candida albicans 3153A*.

- **Antibiotic Controls**

Polymyxin B sulphate, nisin and nystatin were used as positive controls against *E.coli 0111*, *S.aureus NCTC 4163* and *C.albicans 3153A*, respectively. Polymyxin B sulphate (P-1004) and nystatin (N-4503) were sourced from Sigma-Aldrich (St. Louis, MO, USA), and nisin from Aplin and Barrett Ltd, UK.

- **Underlay**

The underlay media for the radial diffusion assay was made with 1% low electroendosmosis agarose and 10mM sodium phosphate, pH 5.5. The media was autoclaved and used for assays at 50^{o}C.

- **Overlay**

Overlay for the radial diffusion assay, to support bacterial growth, was made with 1% agar and 6% trypticase soy broth. For *C.albicans*, the overlay contained 1% agar and 6% Saboraud's medium. The media was autoclaved and used for assays at 50^{o}C.

3.2 Methods

3.2.1 Isolation of Ovine Hemoglobin, its Apoglobins and Peptides

3.2.1.1 Isolation of Ovine Hemoglobin from Blood

Ovine hemoglobin was isolated from fresh whole blood using a red blood cell lysis method adapted from Borenstein et al. (1991).

Four litres of ovine blood was collected directly from the neck of Halal slaughtered sheep. This was immediately mixed with 400ml of 10% sodium citrate, which acts as an anti-coagulant, and stored on ice. The blood was then filtered using cheese cloth to remove any wool, clots or other solid contaminants. In a 1:1 ratio the blood was mixed with 0.83% (w/v) ammonium chloride, resulting in lysis of the red blood cells without affecting the integrity of the white blood cells. The blood and ammonium chloride mixture was then centrifuged at 3000 rpm for 15mins at 4^{o}C (HITACHI himac CR22G2 refrigerated centrifuge) to separate the hemoglobin from the plasma and white blood cells. After the first centrifugation, the plasma layer on top was discarded, more ammonium chloride was added in a 1:1 ratio to the remaining solution, and the centrifugation was repeated.

To ensure the red blood cells were lysed, a drop of the hemoglobin solution was smeared on a glass slide and stained using a Diff-Quick staining kit (Baxter Scientific, Miami, FL, USA). This was then viewed under a microscope to determine if any unlysed red blood cells remained. If so, the ammonium chloride and centrifugation steps were repeated. The fractions were stored at -80^{o}C until required.

3.2.1.2 Determination of Hemoglobin Concentration

In order to reproducibly carry out the peptic digestion of ovine hemoglobin, the hemoglobin concentration of the isolated solution was first required. This was determined by the Bradford assay. First, a 5mg/ml bovine hemoglobin stock standard was made and then diluted with reverse osmosis (RO) water to 0.05mg/ml. Duplicates of dilutions containing 0 to 10ug bovine hemoglobin were made using the diluted standard and RO water to a total volume of 500ul. Bio-Rad (Hercules, CA, USA) protein assay dye reagent concentrate was diluted 1:5 with RO water and 1ml of this was added to each standard. The absorbance of each standard at 590nm was recorded and a standard curve was created using averages of the duplicates. The ovine hemoglobin fraction was diluted in duplicate in the same manner as the standards, until an absorbance was reached that fell within the range of the standard curve. The ovine hemoglobin concentration was then calculated.

3.2.1.3 Acid Acetone Precipitation of Globins

Heme-free globins were obtained by following an acid acetone precipitation method by Acharya & Srinivasulu (2003). The hemoglobin solution was first diluted with water to about 10mg/ml and kept on ice. This solution was added drop-wise to ice-cold acid acetone (pH 3) until the globins were precipitated as a fluffy white product. The precipitated solution was then vaccum filtered, separating the globin precipitate from the heme solution. The precipitate was washed a further three times with acid acetone to ensure the removal of heme, and the product was allowed to air dry.

3.2.1.4 Peptic Digestion of Ovine Hemoglobin

The method for peptic digestion of ovine hemoglobin was adapted from Nedjar-Arroume et al. (2008) and Su et al. (2007b). Hemoglobin solution was diluted to 10mg/ml (1% w/v) using 0.1M sodium acetate, pH 4.5, in the presence and absence of 5.3M urea. Porcine pepsin at a concentration of 1mg/ml was used to digest the hemoglobin in a water bath, at 37^{o}C. Samples were taken at time points: 0mins, 10mins, 30mins, 1hrs, 3hrs, 5hrs and 24hrs.

0.32M disodium tetraborate, pH 12.7, was then added until a pH of 10 was reached, stopping the digestion. The samples were then freeze dried at -80^{o}C and stored at -20^{o}C.

3.2.1.5 Tricine SDS-PAGE

Tricine SDS-PAGE (tricine sodium dodecyl sulphate- polyacrylamide gel electrophoresis) was used to give an estimate of the peptide sizes and quantities (relative to the starting hemoglobin concentration) produced over the course of hemoglobin peptic digestion. This method was selected over normal SDS-PAGE, despite the longer running time, as it provides good separation of small peptides. The method used follows that by Schagger & Vonjagow (1987).

The mini-gel made in a Bio-Rad (Hercules, CA, USA) gel holder and consisted of three layers: resolving gel (bottom), spacer gel (middle) and stacking gel (top). Each layer was prepared as shown in the Table 3.1 and 10ul of TEMED (N, N, N', N'-tetramethylethylenediamine) and 50ul of 10% ammonium persulphate were added immediately before pouring. Each layer was allowed to set before the addition of the next layer. When the stacking gel was poured, a comb was added to form loading wells.

Table 3.1 – Composition of tricine SDS-PAGE gel.

Solution	Resolving gel	Spacer gel	Stacking gel
49.5% T*, 3% C*	-	1.2ml	0.4ml
49.5% T*, 6% C*	2.5ml	-	-
Gel buffer *(3M Tris, 0.3% SDS, pH 8.45)*	2.5ml	2ml	1.3ml
Water	2.5ml	2.5ml	3.3ml
%T	16.5%	10%	4%

*Where 'T' is the total percentage monomer concentration in the gel (acrylamide plus bisacrylamide, w/v), and 'C' is the percentage of monomer that is crosslinker (bisacrylamide).

5mg/ml of the freeze dried digestions were diluted 1:1 with sample buffer (25ml 0.5M Tris-HCl buffer, 20ml glycerol, 40ml 10% SDS, 10ml beta-mercaptoethanol, and 5ml 0.1% bromophenol blue) and heated for 3min at 80^{o}C, then cooled on ice.

Cathode buffer (0.1M tris, 0.1M tricine, 0.1% SDS) and anode buffer (0.2M tris, pH 8.9) were poured into the appropriate compartments of the electrophoresis apparatus, and the gel was inserted (wells faced inwards). 10ul of each sample and 5ul of broad range standard were loaded into the wells. The apparatus was connected to the power source, run at a constant voltage of 100V, and stopped just before the dye front reached the bottom.

The gel was then fixed for 30min (25% isopropanol, 10% acetic acid in deionised water), then stained for 1 hour (1.25g coomassie brilliant blue R250, 242ml deionised water, 242ml methanol, 46ml acetic acid), followed by destaining overnight (7.5% acetic acid, 5% methanol in deionised water).

3.2.2 Purification of Ovine Hemoglobin Apoglobins and Peptides

3.2.2.1 Desalting using Gel Filtration

The digestion mixtures containing urea were desalted by gel filtration (size exclusion chromatography) as a preparation step for RP-HPLC.

Gel filtration apparatus were created by filling 5ml pipette tips with gel filtration media. First, the tip outlets were blocked with glass wool to allow liquid flow without the loss of filtration media. They were then filled with 3ml of swelled Bio-Rad (Hercules, CA, USA) P-10 gel and clamped into position by stands. Freeze dried digest samples were reconstituted in de-ionised water and vortexed. 0.5ml of sample was added to the top of each gel filtration column, without disturbing the resin, and was allowed to run into the resin bed by gravity. Once all of the sample had entered the resin, de-ionised water was then added to the top of each column, acting as an elutent. The liquid passing through the columns was collected in several fractions, and the Bradford assay was carried out to identify where the protein fraction finished. The fractions containing protein were pooled together for freeze drying, and the other fractions containing salt were discarded.

3.2.2.2 Apoglobin and Peptide Purification by RP-HPLC

RP-HPLC was used to: a) separate alpha and beta ovine apoglobins into fractions for antimicrobial testing; and b) obtain ovine hemoglobin pepsin digestion profiles over time and generate enough of each peptide fraction for antimicrobial testing.

First, 10mg of dried apoglobin precipitate was dissolved in 1ml of water. 250ul of this was injected into a semi-preparative column (Phenomenex Jupiter 10u proteo 90A 250x10mm C12) connected to a Dionex RP-HPLC system, scanning at 215nm. Eluent A was water/trifluoroacetic acid at 1000:1 (v/v) and eluent B was acetonitrile/water/trifluoroacetic acid 600:400:1 (v/v). The gradient used for this separation was 0-65% B over 8min, followed by 65-75% B over 17min, at a flow rate of 5ml/min. The fractions were collected and freeze dried at -80^{o}C.

Secondly, an analytical column (Phenomenex Jupiter 4u Proteo 90A, 250mm x 4.6mm C12) was used to determine the digestion profiles at each time point for digestions with and without urea. This method was adapted from Nedjar-Arroume et al. (2008). 1mg of each desalted freeze dried sample was reconstituted with 1ml of deionised water and 100ul was injected for each RP-HPLC run. A gradient of 0-75% B over 45min, followed by 75-90% B over 15min was used, at a flow rate of 1ml/min. The absorbance scan was carried out at 215nm.

To collect fractions for antimicrobial testing, a semi-preparative column was again used (Phenomenex Jupiter 10u proteo 90A 250x10mm C12). 10mg of freeze dried material was dissolved in 1ml of water and 250ul was injected into the RP-HPLC system. The larger column required a larger flow rate of 5ml/min and the peptide retention times were slightly altered. A gradient of 0-25% B over 15min, then 25-60% B over 35 min was implemented and every fraction was collected manually and freeze dried for antimicrobial testing.

3.2.2.3 Determining Peptide Quantities of RP-HPLC Fractions

The quantity of peptide each RP-HPLC fraction from the pepsin digestions was determined by comparing integrated peak area with that of an external standard, with a known concentration.

A synthetic peptide from the beta subunit of ovine hemoglobin was available for use as a standard. The peptide was –FQKVVAGVANALAHKYH-, which corresponds to residues 129-145 of the beta subunit, and had a molecular weight of 1853.1g/mol.

3mg of standard peptide was dissolved in 1ml of deionised water, and decreasing volumes were injected into the semi-preparative column using the same RP-HPLC run conditions as described earlier. The volumes injected were 250ul, 175ul, 100ul and 50ul, which corresponded to 750ug, 525ug, 300ug and 150ug of peptide, respectively. From the masses (ug) of standard peptide and the integrated peak areas (mAU*min) calculated by the Chromeleon 6.50 software, a standard curve was created. The integrated peak areas for each fraction of the semi-preparative RP-HPLC runs were then inserted into the standard curve equation to calculate the mass of peptide in each RP-HPLC fraction. See Appendix 2.2 for calculations.

3.2.3 Antimicrobial Activity Determination

3.2.3.1 Radial Diffusion Plate Assay

The radial diffusion assay is a sensitive method for identifying antimicrobial activity of molecules. In this case it was used to determine the antimicrobial activity of: native hemoglobin, its apoglobins and peptides from hemoglobin digestion. The following method was adapted from Parish et al. (2001).

E.coli 0111 and *S.aureus NCTC 4163* were grown from a single colony overnight in Trypticase Soy Broth (TSB) at 37^{o}C. The cells were then subcultured to obtain cells in mid-logarithmic growth phase by adding 100ul of overnight culture to 10ml of fresh TSB. This was incubated

for an additional 2-4hrs at 37^{o}C depending on the organisms doubling time. *C.albicans* was prepared in the same manner, except grown in Saboraud's Broth.

Next, the cells were spun down by centrifugation at 2000rpm for 5min (Eppendorf 5702 centrifuge) and the supernatant was discarded. The cells were then washed with the starting liquid volume, using 10mM sodium phosphate, pH 5.5, by vortexing to resuspend the cells, followed by centrifugation. This washing step was carried out twice at 2000rpm for 5min.

Bacterial cells were added to 20ml of liquid underlay to give a final concentration of 1x10^{7} CFU/plate, and *C.albicans* was added to give a concentration of 4x10^{6} CFU/plate. The volume of each culture to be added to the underlay were calculated using growth curves (see Appendix 2.3) previously prepared for each organism; using the absorbance of the culture at 600nm, the CFU/ml could be determined.

The liquid underlay was poured into 10x10x1.5cm Lab-Tek square petri dishes and allowed to set. Wells were made in the underlay using a hollow 4.4mm rod. The freeze dried RP-HPLC fractions were each reconstituted with 15ul of 0.01% acetic acid, and 10ul of each was pipetted into a well in the underlay. 10ul of a positive and negative control were added to each plate. The positive controls were: 10ug/ml polymyxin B for *E.coli*, 20 I.U. nisin for *S.aureus*, and 1mg/ml nystatin for *C.albicans*. The negative control was always 0.01% acetic acid as that is what the peptides were reconstituted in.

Peptides were allowed to diffuse through the underlay for 3hrs at 37^{o}C. After this time, 20ml of nutrient overlay was poured on top of the underlay layer and allowed to set. The plates were then incubated at 37^{o}C and zones of clearing were measured after 17hrs.

3.2.3.2 Microtitre Broth Assay

Peptides were serially diluted two-fold across each row of a 96 well polypropylene microtitre plate, in a dilution solution of 0.01% acetic acid plus 0.2% BSA. 50ul of the dilution solution was pipetted into each well followed by the addition of 50ul of peptide into the first well of each row, and mixed. 50ul of this dilution was then transferred to the next well, and so forth for each well down the row, giving two-fold dilutions. Next, 50ul of log-phase cells grown in

Mueller-Hinton broth was added to each well to give a final concentration of $1x10^5$ cells. The plate was incubated overnight at 37^oC and was examined the next day. The MIC was taken as the last well in each row free of any growth (i.e. no pellet or turbidity). This is a method modified by Wu & Hancock (1999).

3.2.4 Identification of Antimicrobial Peptides

3.2.4.1 Mass Spectrometry

Six samples were sent for mass spectrometry analysis at the Centre for Protein Research, Department of Biochemistry, University of Otago, Dunedin. The samples were re-solubilised in 30% (v/v) acetonitrile and 0.1% trifluoroacetic acid in water. 1ul of this was then mixed 1:1 (vol) with matrix (10mg/ml alpha cyano-4-hydroxycinnamic acid (CHCA) dissolved in 65% (v/v) aqueous acetonitile containing 0.1% (v/v) trifluoroacetic acid). 0.8ul was spotted onto a MALDI (matrix-assisted laser desorption/ionisation) plate and air dried.

The samples were analysed in a MALDI tandem time-of-flight analyser set to positive ion mode with 800-1000 laser pulses per sample. The strongest 15-20 precursor ions from each sample underwent MS/MS collision-induced dissociation (CID) analysis. CID spectra were obtained with 2000-4000 laser pulses per selected precursor using the 2kV mode and air as the collision gas at a pressure of 1*E-6 torr.

Peptide identities were acquired by searching the MS/MS data against the Uni-Prot/SWISS-PROT amino acid sequence database, using the Mascot search engine. ‘No enzyme’ was set as a search parameter, as were the modifications, deamidation (D, N) and oxidised methionine, pyroglutamate (E, Q). The precursor mass tolerance threshold was 75ppm, and the maximum fragment mass error was 0.4Da (Centre for Protein Research, 2011).

Chapter 4

Generation of Apoglobins and Peptides from Native Ovine Hemoglobin

4.1 Introduction

This chapter discusses the isolation of ovine hemoglobin from fresh blood as a substrate for obtaining heme-free globins (apoglobins), and peptides by digestion. It also identifies the digestion conditions implemented to obtain peptides with characteristics that may confer antimicrobial activity i.e. short random coiled peptides.

First, methods for isolating ovine hemoglobin from whole blood are discussed, including the properties of blood components, which make separation possible. The characteristics of erythrocytes mean that hemoglobin can be easily obtained at high concentrations by using simple procedures. Erythrocytes are the most numerous cell type in the blood (Campbell & Reece, 2005) and the most dense (Rezapour & Majidi, 2009). Each erythrocyte contains 250 million hemoglobin (Campbell & Reece, 2005) and can be lysed by relatively gentle disruption processes.

Secondly, in order to assess the antimicrobial activity of heme-free ovine hemoglobin subunits, some of the recovered hemoglobin solution was reacted with acidified acetone to remove the heme motifs and precipitate the globins. These apoglobins could later be separated into alpha and beta chains for antimicrobial testing.

Next, the hemoglobin recovered from whole blood was to be digested with the goal of producing highly antimicrobial peptides. A digestion reagent that would result in the formation of short random coiled peptides was first deduced, as each reagent can lead to the generation of different sized peptides, due to varying cleavage specificity. Furthermore, it was hypothesised (based on literature findings) that differing digestion starting substrates (native hemoglobin versus denatured hemoglobin) lead to distinct sets of peptides (Adje et

al., 2011; Choisnard et al., 2002), which may differ in activities. Conditions for obtaining these substrates were investigated.

The digestion reactions were analysed over time, as different degrees of hydrolysis can also result in different bioactive peptides (Choisnard et al., 2002; Su et al., 2007a, 2007b; Zhao et al., 1997 & 1996). This allowed for a comparison of the digestion kinetics of the two ovine hemoglobin starting substrate conformations. The extent of native and denatured hemoglobin digestion was monitored by tricine SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis).

4.2 Results and Discussion

4.2.1 Isolation of Ovine Hemoglobin from Fresh Whole Blood

The objective of this project is to utilise ovine blood from slaughter, with the intention of developing high value antimicrobial products in future. Since fresh ovine blood was readily available in plentiful supply and at no cost, this was the source of hemoglobin used in this research. First, the whole blood cells had to undergo lysis and separation steps to isolate the ovine hemoglobin to be used in the generation of apoglobins and peptides.

In literature, various methods are used to isolate hemoglobin from erythrocytes. Erythrocytes can be isolated by centrifugation of whole blood and then lysed. Alternatively, they may be lysed within the whole blood mixture using reagents that do not affect white blood cell (WBC) integrity, as WBCs contain other antimicrobial components (McCoy, 1988).

Fractionation of blood by centrifugation is based on the relative densities of the cells and components, and is known as a density gradient separation (Rezapour & Majidi, 2009). Figure 4.1 compares the cell numbers, and densities of erythrocytes and the various types of human WBCs, which is the basis of separating of blood components. This figure applies to sheep whole blood cells also, except sheep have smaller and denser erythrocytes than humans (Rezapour & Majidi, 2009). The relative densities of blood components can be further observed in Figure 4.2 where whole blood has been fractionated. Plasma is the least dense layer consisting of mainly water, but also albumin, fibrinogen, immunoglobulins, electrolytes and other substances transported by blood. The buffy coat layer is comprised of a high concentration of WBCs and platelets. Erythrocytes are the densest component of blood, and are present in the highest numbers (Campbell & Reece, 2005). A small amount of the denser WBCs may also be present in the erythrocyte fraction (as shown in Figure 4.1), but in very small quantities in comparison, as there are 1:1000 WBCs to erythrocytes (Campbell & Reece, 2005).

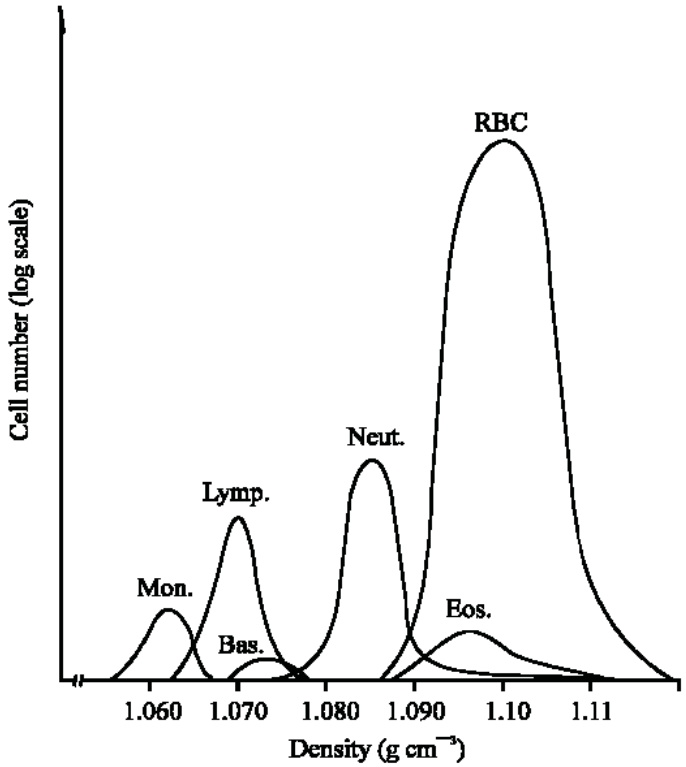


Figure 4.1 - Blood cell number versus density : The basis of density gradient separation of whole blood (Rezapour & Majidi, 2009).

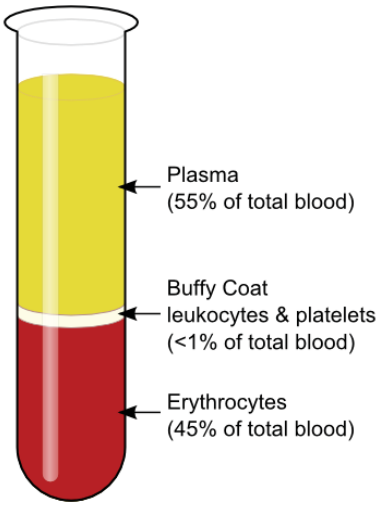


Figure 4.2 - Fractionation of whole blood (Marieb & Hoehn, 2007).

Research groups who used whole blood as a starting material in the generation of hemoglobin peptides utilised a range of physical conditions and reagents to disrupt erythrocyte membranes. Liepke et al. (2003) isolated human erythrocytes from whole blood and used alternating freezing (-80^{o}C) and thawing cycles (4^{o}C) to lyse the cells. Parish et al. (2001) used ice cold water to cause osmotic shock to alligator erythrocytes, releasing hemoglobin. Mak et al. (2000) lysed human erythrocytes using a water-toulene mix.

In this project, ovine hemoglobin was isolated from fresh sheep blood using an isotonic lysis/density gradient separation method. The aim was to effectively separate hemoglobin from whole blood by using minimal steps and reagents. Erythrocytes were lysed within whole blood by 0.83% ammonium chloride (isotonic), at a ratio of 1:1 whole blood to ammonium chloride, and the blood components separated by centrifugation. Figure 4.3 provides a summary of the hemoglobin isolation process from whole blood. Aside from the simplicity of this method, it is fast, and does not require initial separation of red and white blood cells as ammonium chloride has a negligible effect on the integrity of WBCs (McCoy,

1988), unlike water or NaCl (at non-physiological concentrations). When using water or NaCl as erythrocyte lysis agents within whole blood, another buffer must then be added to return the solution back to isotonicity, in order to prevent WBC lysis (Anderson, 2005). Moreover, ammonium chloride has a reputation as an erythrocyte lysis agent as it is the key ingredient in many commercial erythrocyte lysis buffers (Chernyshev et al., 2008).

Typically, a solution must be hypotonic for erythrocytes to lyse. However, interestingly it has been found that an ion exchange cycle across the erythrocyte membrane affects the swelling of erythrocytes in an isotonic ammonium chloride solution (Chernyshev et al., 2008), making isotonic lysis possible. This process is governed by the Jacobs-Stewart cycle.

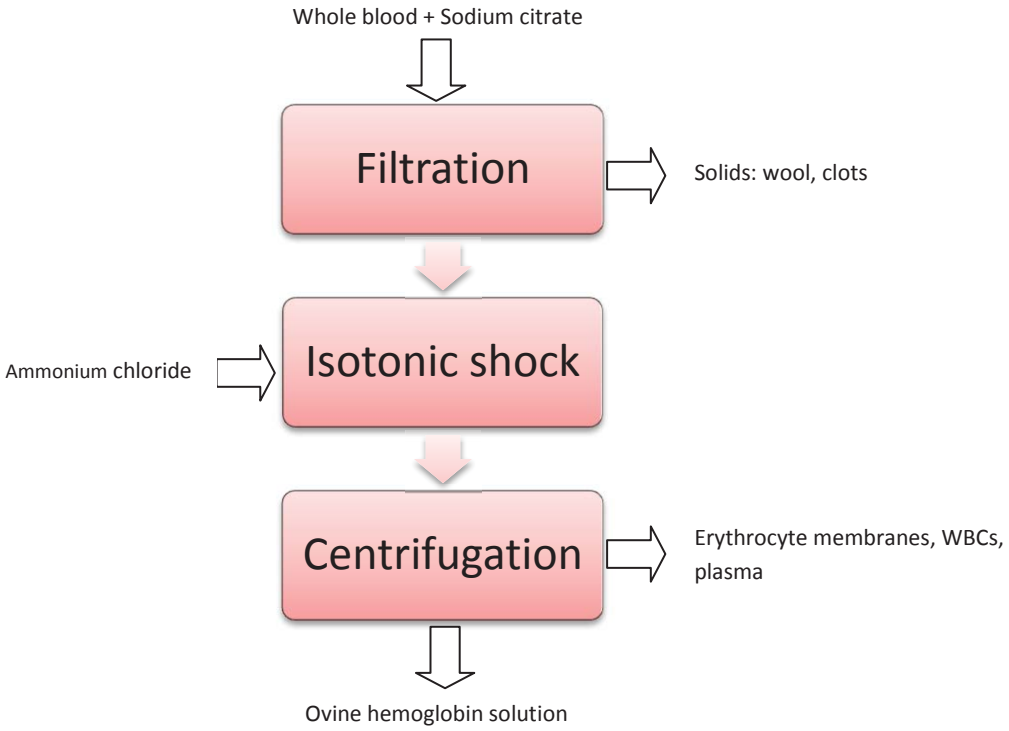


Figure 4.3 - Summarised method for ovine hemoglobin isolation from whole blood.

The extent of erythrocyte lysis was monitored by examining stained blood samples under the microscope after each isotonic lysis and centrifugation, as shown in Figure 4.4. The ammonium chloride lysis step had to be carried out twice to lyse the majority of erythrocytes. Another alteration to the method was the increase in centrifugation speed from 2000rpm to 3000rpm. This was necessary because a speed of 2000rpm did not give adequate separation of erythrocytes from plasma. A lot of un-lysed erythrocytes had

remained in the plasma layer, as characterised by the opaque dark red colour and the inability to distinguish between plasma and cell fractions. The lysis of erythrocytes was characterised by the change in solution colour, from opaque red to translucent red.

The ovine hemoglobin concentration of the final solution was then determined by a Bradford assay, using bovine hemoglobin as a protein standard (Refer to Appendix 1.1).

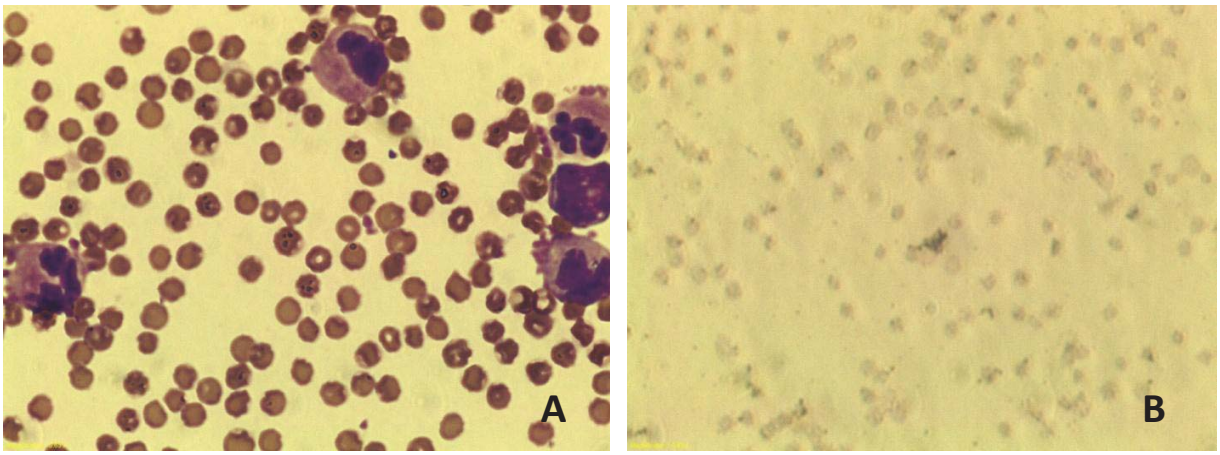


Figure 4.4 - Oil immersion microscopic images of ovine blood cells stained with Diff-Quick (400x magnification). (A) Whole ovine blood, showing unlysed erythrocytes and the presence of white blood cells, (B) Lysed erythrocytes after two ammonium chloride treatments and centrifugation. Membranes remain.

4.2.2 Preparation of Ovine Hemoglobin Apoglobins by Acid Acetone Precipitation

As stated by Mak et al. (2008 & 2000), when hemoglobin is deprived of heme (apo-) it will partially unfold and dissociate into subunits that possess antimicrobial activity not present in the native tetramer.

This theory was to be tested for ovine hemoglobin, as no known publications have discussed the antimicrobial activities of native ovine hemoglobin or its apoglobins.

Mak et al. (2000) prepared human apoglobins for antimicrobial testing by acidic acetone precipitation. This appears to be a very common method for separating heme from globins. The strongly acidic conditions and the concentrated organic solvent work together to dissociate heme and precipitate the globins. The low pH correlates to a reduction in protein helical content (secondary structure), which results in heme release, whilst the organic

solvent affects tertiary structure, which in turn reduces the stability of the heme binding pocket (Wang et al., 2010).

Following a method adapted from Acharya & Srinivasulu (2003), ovine hemoglobin solution was added slowly to ice-cold acidified acetone and stirred until fluffy white protein precipitates were observed. The solution was then passed through a vacuum filter and the precipitate was retained, and washed twice more with acid acetone to ensure heme removal (see Figure 4.5).

After drying, the globin powder was dissolved in distilled water, giving a strongly acidic solution due to the presence of hydrochloric acid (Anson & Mirsky, 1931 & 1930). The apoglobins could then later be separated into alpha and beta fractions by RP-HPLC.

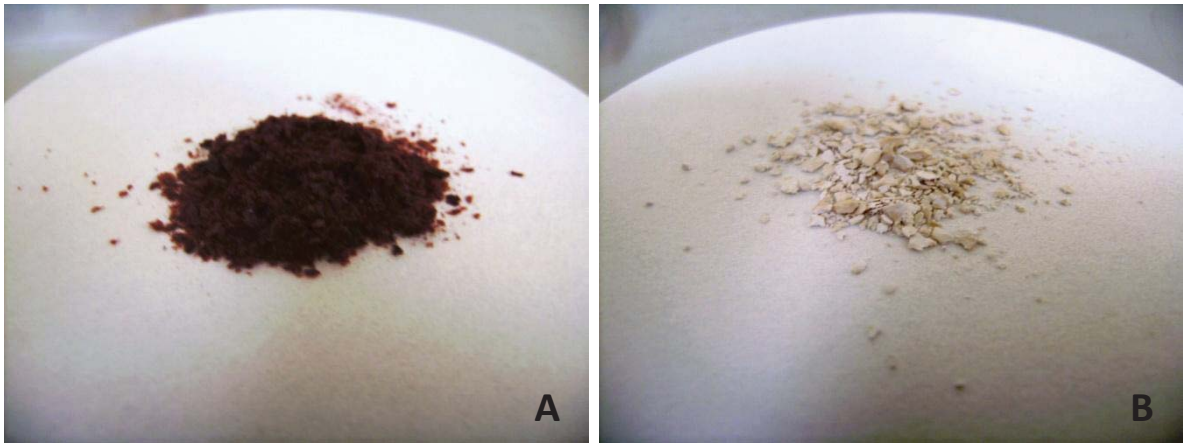


Figure 4.5 – Ovine hemoglobin heme removal and globin precipitation by acid acetone. (A) Native ovine hemoglobin, (B) Acid acetone precipitated ovine apoglobins.

4.2.3 Generation of Peptides from Ovine Hemoglobin Digestion

Bioactive peptides can be produced *in vitro* by the use of different chemicals and enzymes on functional proteins. The enzymes responsible for the generation of bioactive peptides *in vivo*, however, are largely unknown.

Different digestion agents target different peptide bonds, resulting in unique sets of peptides. Therefore, before selecting a suitable enzyme or chemical for hemoglobin digestion in this project, the desired peptide characteristics that confer antimicrobial activity were first identified.

The majority of research groups investigating hemocidins have produced AMPs that are at least 30 amino acids long, using various enzymes or chemicals (Daoud et al., 2005; Mak, 2008; Mak et al., 2000; Parish et al., 2001). However, Nedjar-Arroume et al. (2008) carried out pepsin digestion of bovine hemoglobin, which resulted in extremely active peptides as small as 5 residues long. These peptides had fewer hydrophobic residues in comparison to classic AMPs and were random coils/lacked secondary structure (Nedjar-Arroume et al., 2008). Furthermore, it was later reported that the minimal sequences from the bovine hemoglobin alpha and beta chains required to exert antimicrobial activity also had the strongest activities (Catiau et al., 2011a, 2011b) . These were just 3 amino acids long. As a consequence of this knowledge, it was hypothesised that small random coiled fragments from the digestion of ovine hemoglobin are likely to have strong antimicrobial activity.

Pepsin appears to be the most commonly utilised enzyme for *in vitro* digestion of functional proteins to produce bioactive peptides. This may be due to the knowledge that many bioactive peptides are released from food proteins in the stomach, where they are most likely generated by pepsin (Mak, 2008). Bioactive peptides that have been generated *in vitro* by pepsin digestion include opioid, antimicrobial, antihypertensive, antithrombotic peptides (Su et al., 2007a) and hemocidins. Other enzymes and chemicals noted in literature in the generation of hemocidins include trypsin, arginine-C (Arg-C) (Mak et al., 2000) and cyanogen bromide (CNBr) (Mak et al., 2000; Parish et al., 2001). Arg-C and CNBr cleavage result in fewer, much larger, fragments than pepsin for the same amount of digestion time. This is due to the fact that they have high specificity in comparison to pepsin (pepsin is a non-specific enzyme), cleaving at only the C-terminal of arginine and methionine residues, respectively, and there are only a few of these residues in the hemoglobin sequence. Therefore, Arg-C and CNBr were excluded as potential digestion reagents for this research. Although trypsin digestion fits the criteria for the generation of small fragments, the resulting peptides had little or no activity according to Mak et al. (2000). Furthermore, trypsin cannot hydrolyse peptide bonds in native hemoglobin (Anson & Mirsky, 1934), which is a starting substrate in this project; the protein must be denatured. Taking into account these factors and the recent successes reported in literature in obtaining short highly active hemocidins from hemoglobin pepsin digestion, pepsin was the obvious agent for ovine hemoglobin digestion.

Next, the conditions required for hemoglobin pepsin digestion were determined. Digesting hemoglobin in its native state and in its denatured state gives different sets of peptides due to different kinetics (Choisnard et al., 2002; Su et al., 2007a). Therefore, the pepsin digestion of both of these substrates was to be followed in order to identify which digestion results in a high yield of short random coiled peptides. Two factors, pH and the use of the denaturing agent (urea) were utilised to achieve these different forms of starting substrate. First, the pH of the digestion solution had to be acidic for pepsin to be active; pH 2 is optimal for pepsin activity (Su et al., 2007b). However, at a pH below 4.0, heme groups detach from their binding sites (Wang et al., 2010; Zhao et al., 2010), and ionisation of globin chains occurs resulting in subunit dissociation (Zhao et al., 2010). Furthermore, a pH as low as 2 will also cause hemoglobin denaturation (Su et al., 2007a; Zhao et al., 1996), which is another unwanted characteristic in the first experimental condition, where the starting substrate is to be native hemoglobin. Therefore, pH 4.5 was selected to carry out digestion experiments in this project, as a compromise between pepsin activity and maintaining the integrity of native hemoglobin.

To achieve denatured hemoglobin as a starting substrate, 5.3M urea (Nedjar-Arroume et al., 2008) was utilised under the same acidic conditions. Elbaum & Herskovits (1974) report that in low urea concentrations human hemoglobin is dissociated into alpha-beta dimers with little denaturation, and above 5M urea almost all hemoglobin molecules are dissociated into monomers and are denatured. Urea is also an ideal denaturant as it has been found to have no denaturing effects on pepsin, and pepsin is many times more soluble in urea than in water (Steinhardt, 1938). Furthermore, urea keeps denatured hemoglobin soluble in the reaction solution (Steinhardt, 1938).

To follow the generation of new peptide sequences and their concentration changes, the hemoglobin digestion was stopped after time points 0, 10, 30min, 1, 3, 5 and 24hrs, by raising the pH to 10 to inactivate the pepsin. Originally, this was carried out by the addition of ammonium hydroxide, as used by Su et al. (2007b). However, it was found that this was not very user-friendly due to its pungent smell and the potential effect on the life of the freeze drier. All following digestions were stopped by the addition of 0.32M, pH 12.7 disodium tetraborate, following the method by Nedjar-Arroume et al. (2008).

4.2.4 Separation of Ovine Hemoglobin Peptides by Gel Electrophoresis

To determine the extent of ovine hemoglobin pepsin digestion over time and compare digestion kinetics of the two starting substrates, SDS-PAGE was carried out on the hydrolysates from each time point.

SDS-PAGE is used to separate proteins and peptides on the basis of their molecular mass. In particular, tricine-SDS-PAGE is designed for resolving proteins smaller than 30kDa (Schaegger, 2006), which is applicable to the peptides generated here.

As shown by Figure 4.6, unfolding and denaturation of native ovine hemoglobin by SDS and sample heating resulted in ovine hemoglobin monomers present on the gel, rather than the 66kDa tetramer (Huisman et al., 1965); two bands can be seen at 16.07kDa and 15.17kDa, which correspond to ovine hemoglobin beta and alpha subunits, respectively. In both gels, the intensity and thickness of these bands decreased over time as they were digested into smaller peptides by pepsin; this occurred much more rapidly in the case of the urea treated hemoglobin. Significant digestion of the urea treated hemoglobin (Figure 4.6 (B)) had already occurred at 10min, whereas it took 30min to see any peptide product from the digested native hemoglobin (Figure 4.6 (A)). Furthermore, in agreement with the literature, as well as the difference in reaction rate, Figure 4.6 suggests that the pattern of digestion kinetics of the two starting substrates is quite different. Three bands consisting of different sizes of peptide product were observed in the early stages of digesting urea treated hemoglobin, as opposed to one for the native form. This is likely due to the greater accessibility of pepsin to the protein peptide bonds when hemoglobin was in its denatured state. It appears that for the urea treated hemoglobin some peptides were generated, which were then further digested into smaller fragments. The sizes of the final peptides for both digestion conditions were below that of the smallest marker (Aprotinin), which is 6.5kDa. It can be seen that there was another band at approximately 31kDa, which was also degraded over time. This most likely corresponds to ovine hemoglobin dimers, which have a molecular weight of 31kDa also.

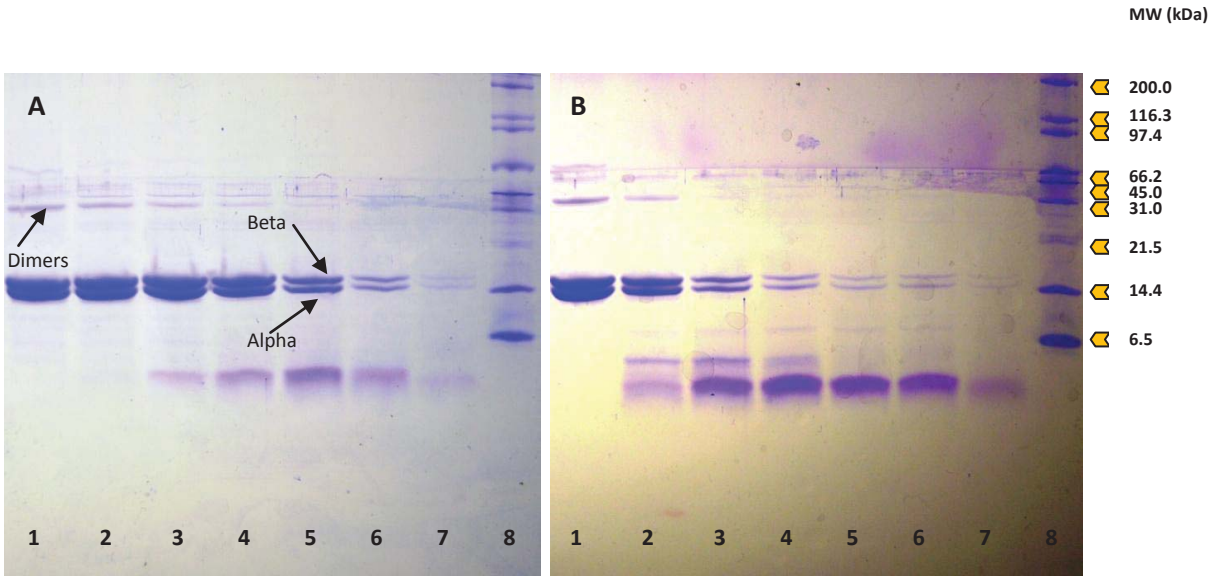


Figure 4.6 - Tricine SDS-PAGE gels of ovine hemoglobin digestions over time. (A) Pepsin digestion of native ovine hemoglobin, (B) Pepsin digestion of urea treated ovine hemoglobin. Lane: 1) 0min, 2) 10min, 3) 30min, 4) 1hr, 5) 3hr, 6) 5hr, 7) 24hr and 8) Protein standard marker.

4.3 Conclusions

Ovine hemoglobin was successfully isolated from the erythrocytes of fresh whole sheep blood. This was achieved by lysing erythrocytes within the whole blood mixture, using a simple isotonic ammonium chloride lysis and centrifugation procedure. A couple of changes to the method were implemented; two ammonium chloride treatments were required to lyse the majority of erythrocytes; and the centrifugation speed was increased to provide better separation of erythrocytes from the plasma layer.

The ovine hemoglobin was then used to obtain apoglobins and peptides for antimicrobial testing. Ovine heme-free globins were precipitated as a white product by three acidic acetone washes. These were to be later separated into alpha and beta globins fractions by RP-HPLC.

Based on literature evidence that suggests high antimicrobial potency of short random coiled peptides, digestion conditions were selected to obtain peptides with these characteristics. It was concluded that pepsin is an ideal digestion reagent to achieve this based on its reported ability to generate bioactive peptides, and its non-specificity, allowing for digestion into very short fragments, which is not possible with other enzymes or chemicals. Furthermore, it was identified that pH and urea concentration effect the conformation of hemoglobin. Manipulation of these factors allowed for different starting substrates, which were cleaved following different kinetics, producing unique peptide sets. It was hypothesised that these sets of peptides may differ in their antimicrobial effectiveness towards microorganisms. An acidic pH of 4.5 was selected for hemoglobin digestion to ensure both pepsin activity and native conformation was maintained. The addition of 5.3M urea to the reaction allowed for the second digestion substrate-denatured hemoglobin.

The extent of hemoglobin digestion over time was monitored by tricine SDS-PAGE, which provides a high resolution for proteins/peptides lower than 30kDa. It was clear that in the presence of urea, hemoglobin is digested by pepsin much more rapidly, and the two starting substrates appeared to be digested following different kinetics.

Chapter 5

Purification of Ovine Hemoglobin Apoglobins and Peptides

5.1 Introduction

This chapter discusses the purification of apoglobins, and peptides from the pepsin digestions of ovine hemoglobin performed in chapter 4. It also aims to identify which conditions (digestion time and starting substrate) are required to produce high yields of short peptides.

First, heme-free denatured globins from the acid-acetone precipitation of native ovine hemoglobin were separated into alpha and beta chain fractions by RP-HPLC, to be later tested for antimicrobial activity.

For the analysis of ovine hemoglobin peptides, RP-HPLC was first carried out on an analytical column in order to produce digestion profiles over the course of the reaction, and to allow comparison of the pepsin digestion kinetics between non-urea and urea conditions. As mentioned in chapter 4, these conditions affect the starting substrate conformation. Furthermore, it was hypothesised based on the pepsin digestion kinetics of bovine hemoglobin (Adje et al., 2011; Choisnard et al., 2002; Su et al., 2007a, 2007b; Zhao et al., 1996) that ovine hemoglobin would be digested in the same way, following the 'one-by-one' mechanism for digestion of native hemoglobin as a substrate, and the 'zipper' mechanism for the urea treated protein.

Lastly, semi-preparative RP-HPLC was carried out on the pepsin digests to produce adequate amounts of peptide for antimicrobial testing.

5.2 Results and Discussion

5.2.1 Separation of Ovine Hemoglobin Apoglobins by RP-HPLC

250ul of the acidic apoglobin solution at 10mg/ml was injected into a semi-preparative RP-HPLC column to separate the alpha and beta apoglobins into their distinct fractions. This produced sufficient amounts of each apoglobin for antimicrobial testing.

Figure 5.1 shows the separation of the globin chains (lacking heme) as two fractions on the RP-HPLC chromatogram. The first apoglobin fraction eluted at approximately 14.5min, and the second at 17.5min, which correspond to 69% and 70.5% buffer B respectively (see methods for buffer composition).

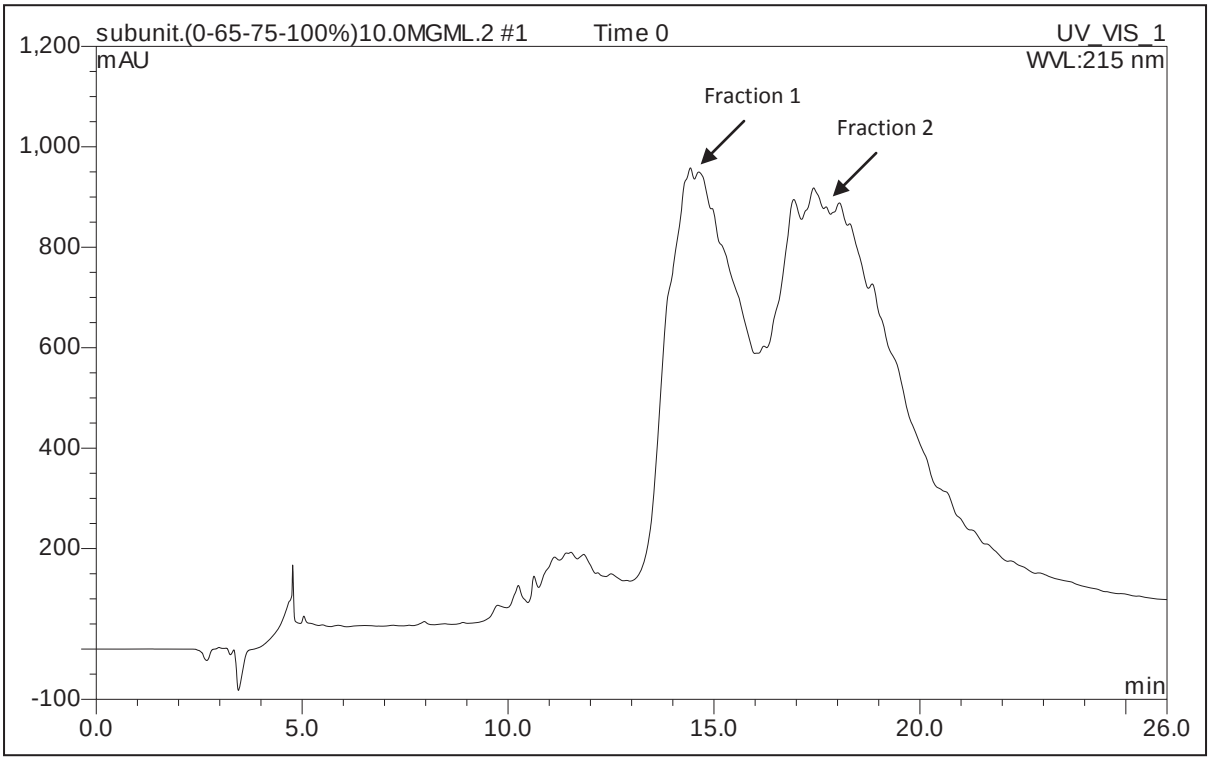


Figure 5.1 – Semi-preparative RP-HPLC chromatogram for the separation of ovine hemoglobin apoglobins.

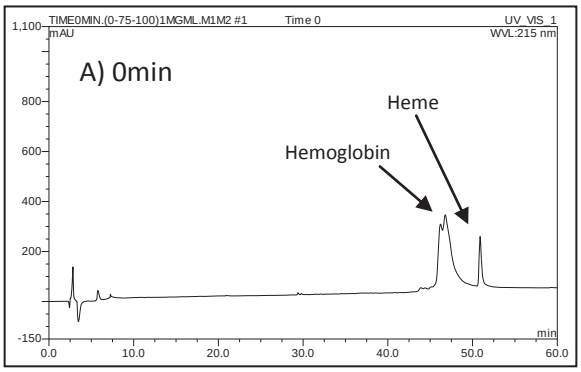
Typically, it is understood that the conditions of RP-HPLC are strong enough to dissociate hemoglobin subunits and release heme (Wajcman, 2003) i.e. RP-HPLC alone will result in apoglobins. However, after experimentation with varying RP-HPLC gradients over extended

time periods, it was found that this was not the case for ovine hemoglobin. Therefore, the use of the acid acetone globin chain precipitation method was an essential preparative step for RP-HPLC in order to obtain the apoglobins. This may suggest greater stability of the ovine hemoglobin tetramer in comparision to other hemoglobin species. The separation, however, may be more effective on a column with different characteristics. In literature a C4 column is commonly used to separate hemoglobin chains.

5.2.2 Ovine Hemoglobin Pepsin Digestion Profiles from RP-HPLC

An analytical RP-HPLC column was used to obtain digestion profiles for each time point, for the urea and non-urea treated hemoglobin pepsin digests. Initially a gradient of 0-100% B was run over 40min to identify the buffer concentration range where the peptides elute. The last peptide eluted before 75% B, while the undigested hemoglobin and heme presented as two peaks after this concentration. Therefore, the gradient was optimised to 0-75% B over 45min, followed by 75-90% over 15min to allow for good peptide fraction separation (see methods for details).

The freeze dried digests from each time point to be profiled were reconstituted to 1mg protein/ml. Because the urea treated hemoglobin digests contained a large quantity of salt (urea), a lot more sample had to be weighed to give the same protein/peptide concentration as the non-urea digestion samples. It was necessary to have the same protein/peptide concentration from each digestion condition and time point injected into the RP-HPLC column in order to produce comparable profiles between the two. Figure 5.2 compares the digestion profiles of the two starting substrates over time.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Time 0
UV_VIS_1
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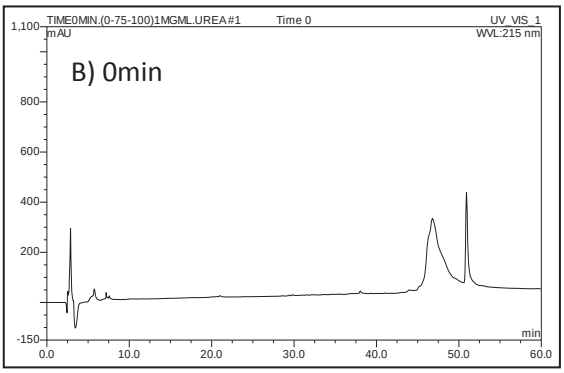
mAU
A) 0min
Heme
Hemoglobin
min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UV_VIS_1
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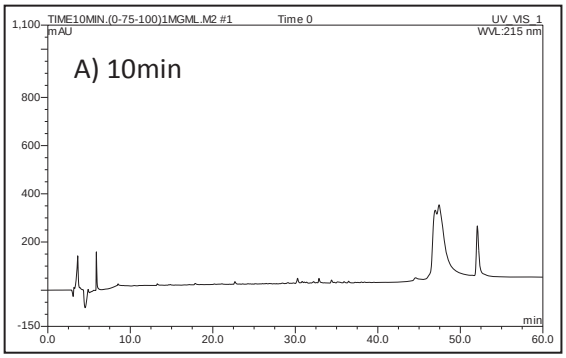
mAU
B) 0min
min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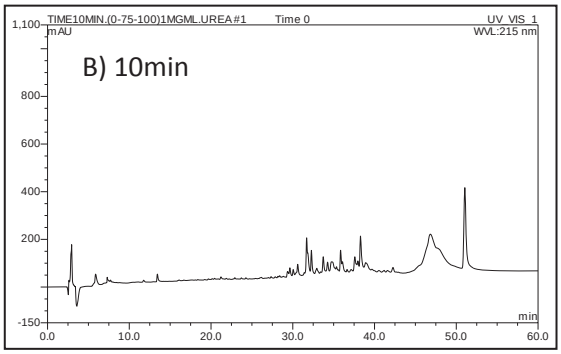
Time 0
UV_VIS_1
WVL:215 nm
mAU
A) 10min
min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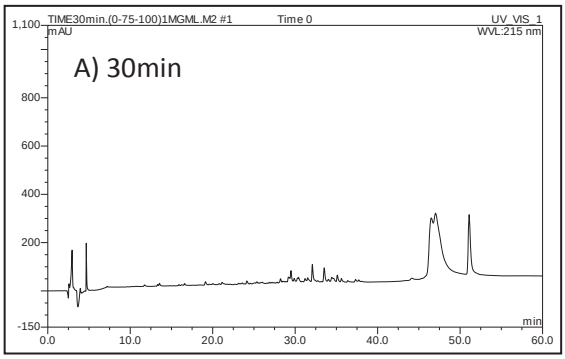
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UV_VIS_1
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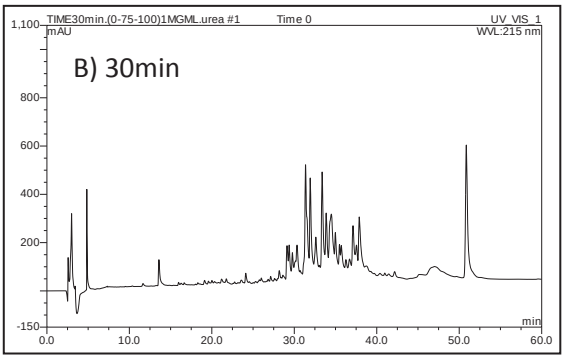
mAU
A) 30min
min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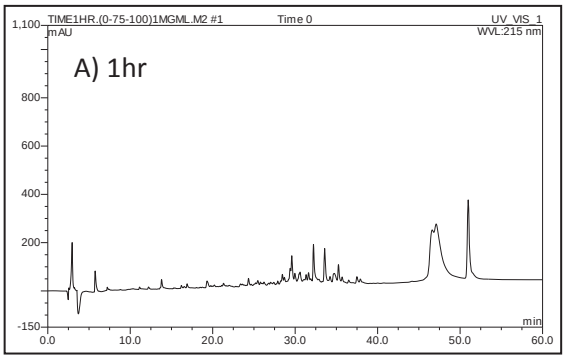
Time 0
UV_VIS_1
WVL:215 nm
mAU
B) 30min
min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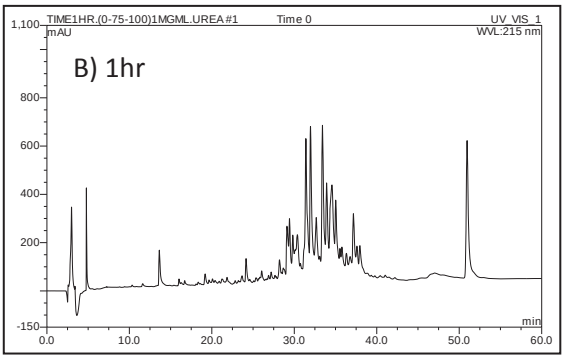
Time 0
UV_VIS_1
WVL:215 nm
mAU
A) 1hr
min


TIME1HR.(0-75-100)1MGML.UREA #1
Time 0
UV_VIS_1
WVL:215 nm
mAU
B) 1hr
min

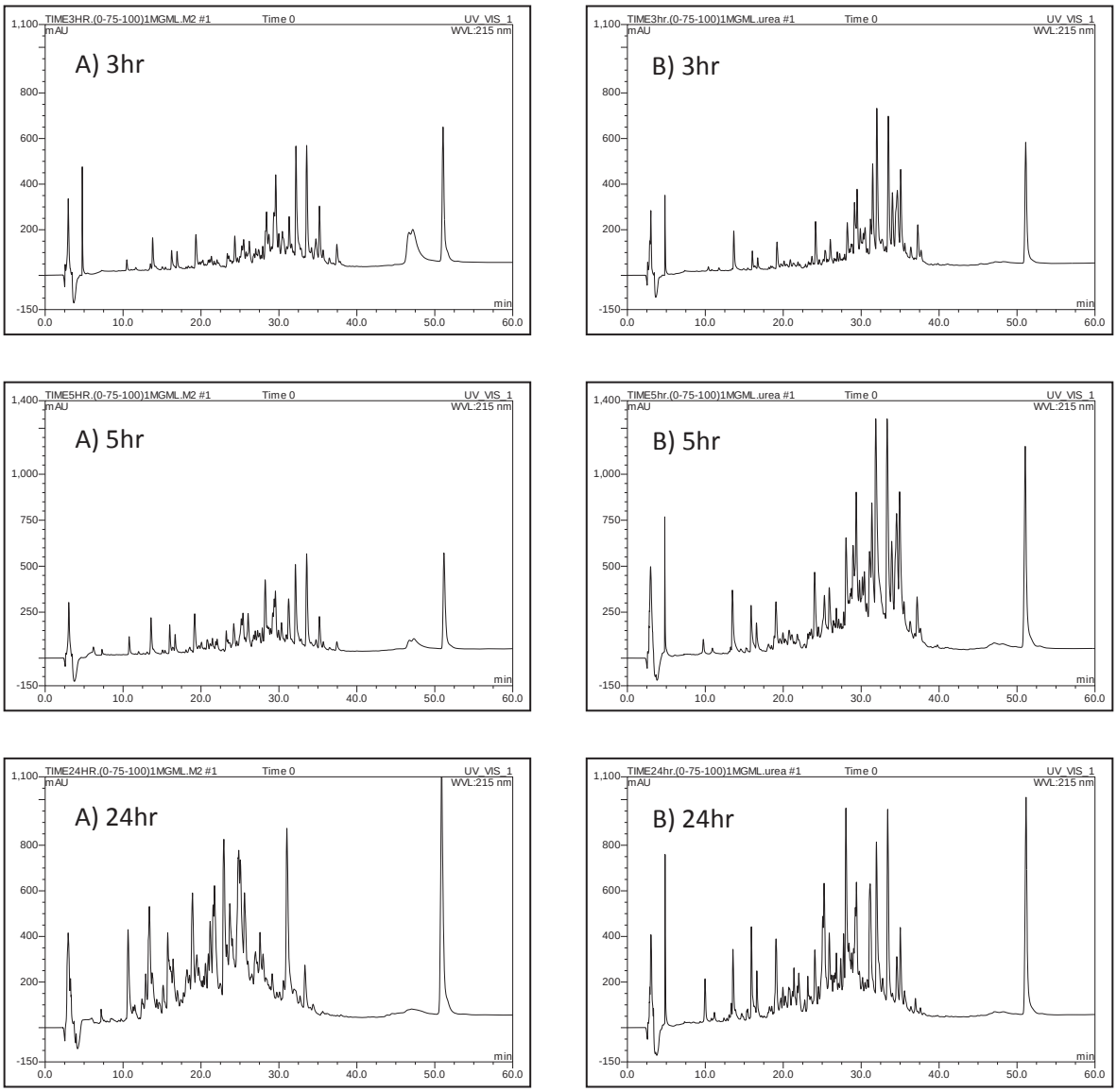


Figure 5.2 - Analytical RP-HPLC profiles of pepsin digested ovine hemoglobin over 24hrs. (A) Ovine hemoglobin digested by pepsin in sodium acetate buffer. The starting substrate is native hemoglobin, (B) Ovine hemoglobin digested by pepsin in sodium acetate buffer with 5.3M urea.

It is noticeable that as time progresses, the peak corresponding to the parent protein, ovine hemoglobin, gradually decreases as various peptides are liberated by pepsin digestion. In agreement with the tricine SDS-PAGE gels, the RP-HPLC profiles suggest two different digestion mechanisms exist; one for pepsin digestion of ovine hemoglobin in the presence of urea, and another without.

5.2.2.1 Kinetics of Native Ovine Hemoglobin Pepsin Digestion

It is said that when native hemoglobin is digested by pepsin the kinetics follow the 'one-by-one' mechanism; one hemoglobin molecule is hydrolysed at a time directly into small final peptide products (Adje et al., 2011; Choisnard et al., 2002). This means that few intermediate products are likely to result under these kinetics. This is in fact the case for the pepsin digestion of native ovine hemoglobin in this research, as seen in Figure 5.2 (A). It can be observed that the hydrolysate is comprised of only the parent protein, hemoglobin, and final peptide products, whose concentration continually increases overtime until all the parent protein has been completely consumed i.e. existing peptide fractions continually increase in height as native hemoglobin is digested rather than resulting in the formation of new fractions/peptides at later time points.

It can be observed from the earliest RP-HPLC profiles (Figure 5.2) that hydrophobic peptides are generated first (corresponding to spectra on the right-hand side of the chromatograms) and increase more rapidly in concentration than less hydrophobic peptides. This agrees with literature concerning pepsin digestion of native bovine hemoglobin (Choisnard et al., 2002; Su et al., 2007b). This is due to the fact that pepsin preferentially cleaves hydrophobic regions of protein, particularly at the C-terminal of F (phenylalanine), L (leucine) and E (glutamic acid) residues (Su et al., 2007b).

Interestingly, at a digestion time between 5hrs and 24hrs, the retention time for the last peptide eluted (for the non-urea condition) shifts from 37.5min to 33.5min, suggesting that peptides within this retention time period are further degraded with a corresponding increase in the concentration of hydrophilic peptides. This only occurs when native hemoglobin has been digested and pepsin moves on to preferentially digest the hydrophobic peptides that have been previously generated.

Furthermore, Su et al. (2007b) found that under this 'one-by-one' mechanism of digestion, the N- and C- terminals of both bovine hemoglobin subunits were the first to be digested by pepsin; from residues 1 to 46 and 99 to 141 of the alpha chain, and 1 to 40 and 105 to 145 of the beta chain. These regions were identified by sequencing the peptides produced throughout the digestion, and recognising that the peptides generated within the first 10min

were largely from the terminal regions. The group then stated that this digestion behaviour is understandable, as pepsin preferentially cleaves hydrophobic regions, and the terminal ends of both subunits are shown to be more hydrophobic than many other protein regions by hydrophobicity plots (see Figure 5.3). Also, the terminal regions are the most accessible to pepsin, which means that they are digested earlier than regions in the hydrophobic core which are not accessible at the start of hydrolysis, although they may have equal or greater hydrophobicity. Comparing the bovine hemoglobin hydrophobicity plots with the ovine hemoglobin plots in Figure 5.3 suggests that this order of digestion for native bovine hemoglobin is likely applicable to native ovine hemoglobin also, as their hydrophobicities are near identical. This makes sense as their sequences are highly conserved, as shown in the literature review.

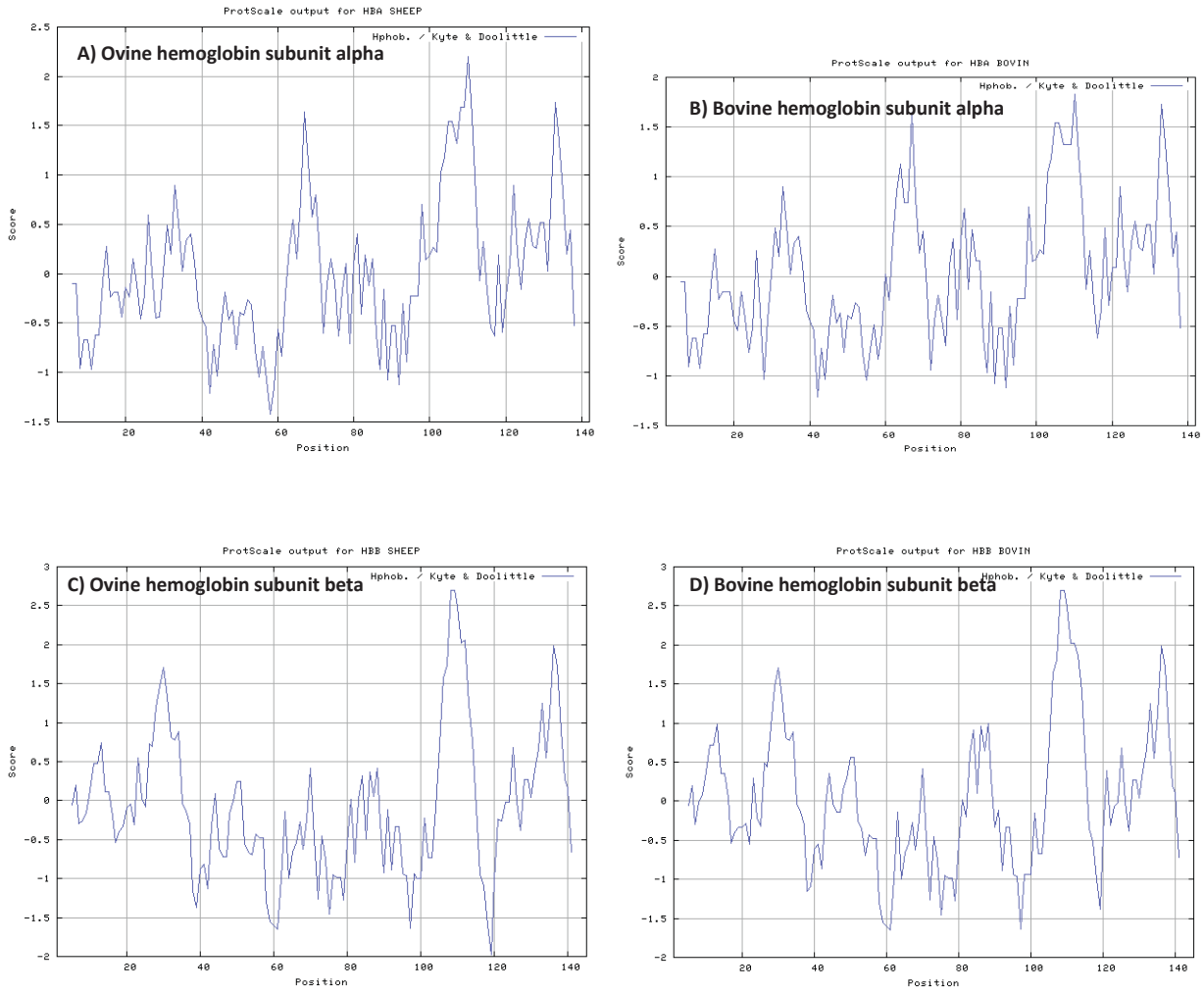


Figure 5.3 - Kyte & Doolittle hydrophobicity plots of ovine and bovine hemoglobin subunits. (A) Ovine hemoglobin subunit alpha, (B) Bovine hemoglobin subunit beta, (C) Ovine hemoglobin subunit beta, (D) Bovine hemoglobin subunit beta (http://web.expasy.org/protscale/).

By looking at the hydrophobicity plots it is noticeable that the centres of the ovine and bovine beta chains are relatively hydrophilic in comparison to the rest of the chain. It is therefore expected that the centre of the beta subunit would be the last region to be digested by pepsin. This is supported by Su et al. (2007b) who stated that at the end of the native bovine hemoglobin pepsin digestion, peptides formed from the alpha chain covered 100% of its total sequence, whereas peptides from the beta chain only covered 76% of the total beta sequence, with peptides from the centre of the beta chain being largely absent. The couple of peptides which did originate from the centre of the beta chain did so due to the non-specificity of the pepsin enzyme.

5.2.2.2 Kinetics of the Pepsin Digestion of Urea Treated Ovine Hemoglobin

In the literature, bovine hemoglobin has been denatured in preparation for pepsin digestion by the presence of concentrated urea or hydrochloric acid (final pH 2). Pepsin digestion of denatured bovine hemoglobin leads to rapid removal of hemoglobin from the hydrolysate (within a couple minutes) as peptides are generated (Choisnard et al., 2002; Su et al., 2007a; Zhao et al., 1996). This is known as the 'zipper' mechanism, whereby intermediate peptide products are formed immediately from the proteolysis of the denatured parental protein, followed by slower digestion of the intermediates into final peptide products (Adje et al., 2011; Choisnard et al., 2002). It is thought that the initial stage of this reaction (conversion of denatured hemoglobin into intermediates) occurs quickly as denaturation of hemoglobin exposes the hydrophobic core of the protein for digestion by pepsin.

By comparing the RP-HPLC profiles of the two digestion conditions in this research (Figure 5.2), it can be seen that the presence of urea has had a marked increase on the rate of conversion of hemoglobin into peptides. As a consequence of the immediate exposure of more hydrophobic regions to pepsin, hydrophobic peptides appear more rapidly and in greater concentrations than for the digestion of native hemoglobin. In the presence of urea, the majority of the parent protein has been digested within 1hr, whereas under the non-urea condition this takes over 5hrs.

Like the pepsin digestion of urea or acid treated bovine hemoglobin (Choisnard et al., 2002; Zhao et al., 1996), it was expected that ovine hemoglobin digestion in the presence of urea would follow the same 'zipper' mechanism of digestion. This was hypothesised based on the high conservation of amino acid sequence and hydrophobicity between the two hemoglobins.

However, this research utilised the same digestion conditions as Choisnard et al. (2002) who digested denatured bovine hemoglobin with pepsin, and the result was ovine RP-HPLC profiles that were different than expected (Figure 5.2 (B)). By viewing the result of Choisnard et al. (2002) in Figure 5.4 you can clearly see that all the denatured bovine hemoglobin has been converted into intermediates or final products within 2.5min - very rapid in comparison to the time required for the disappearance of urea treated ovine hemoglobin from the hydrolysate. Furthermore, following the 'zipper' mechanism, it can be seen that some of the bovine peptides formed early on (intermediates) are further digested into smaller peptides, corresponding to decreases in existing peak heights/peptide concentrations and the formation of new peaks. However, in the ovine profiles, after careful examination, only a few intermediates can be seen and for the most part, peak height/peptide concentration continually increases as in the case of the 'one-by-one' mechanism.

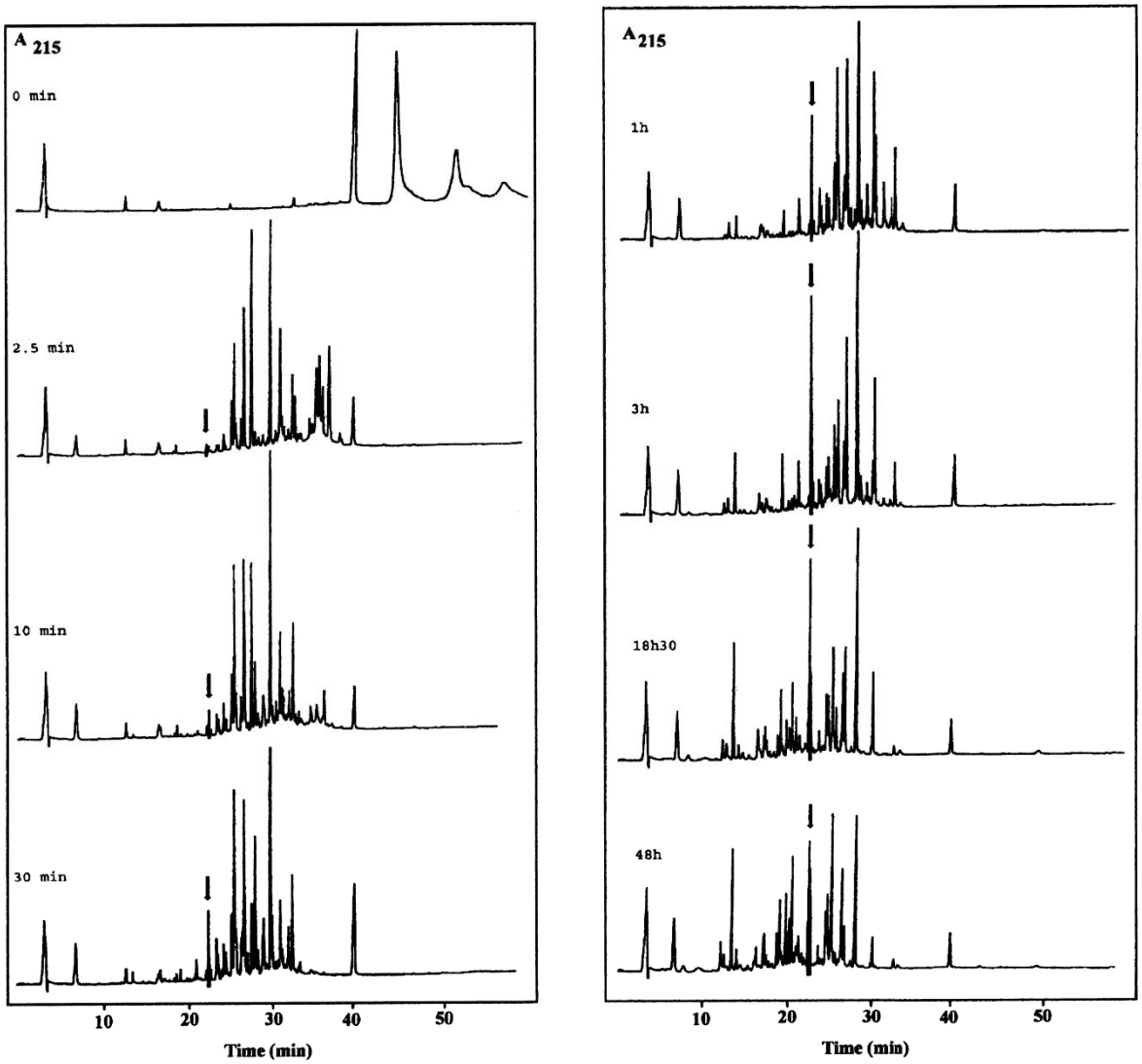


Figure 5.4 - RP-HPLC of urea denatured bovine hemoglobin digested with pepsin, demonstrating the 'zipper' mechanism (Choisnard et al., 2002).

The digestion of ovine hemoglobin in the presence of urea appears to follow a mechanism that has characteristics of both native and denatured hemoglobin pepsin digestion. This suggests that the ovine hemoglobin was not fully denatured by 5.3M urea. Increasing the pepsin concentration may increase the rate of conversion of protein to peptides; however, it would unlikely change the kinetics pattern.

Because a large proportion of the hemoglobin sequence is conserved between the ovine and bovine species, it was assumed that since 5.3M causes denaturation of bovine hemoglobin (Choisnard et al., 2002; Nedjar-Arroume et al., 2008) that this would also be the fate of ovine hemoglobin. As mentioned in chapter 4, in regard to human hemoglobin stability in urea

solutions, Elbaum & Herskovits (1974) state that the hemoglobin tetramer is dissociated into its monomers before the majority of denaturation is able to occur. Moreover, after further reading it was found that Wu and Yang (1932) showed that the sheep hemoglobin tetramer has great stability, demonstrating that cow and horse hemoglobin dissociate in 2 to 6M urea, and sheep and dog hemoglobin do not (Simko & Kauzmann, 1962; Steinhardt, 1938). These facts combined may suggest that ovine hemoglobin does not dissociate into monomers in 5.3M urea, and therefore far less denaturation occurs than what was originally expected. This lack of dissociation is also evident in the RP-HPLC profiles (Figure 5.2); ovine hemoglobin is observed as a single protein peak, plus heme, whereas bovine hemoglobin is present as heme plus two fractions corresponding to the alpha and beta apoglobins (Figure 5.4). As mentioned earlier, ovine hemoglobin could not be separated into its globins by altering the RP-HPLC gradient (on this column at least).

Perhaps a higher urea concentration will be adequate to denature ovine hemoglobin, or may be denaturation of ovine hemoglobin in acid at pH 2 should be considered. Su et al. (2007a) and Zhao et al. (1996) carried out digestion experiments on bovine hemoglobin denatured under acid conditions, pH 2. In this case, after the reaction has been stopped by increasing the pH, the supernatant and precipitate are separated by centrifugation. This method likely results in more peptide losses, however.

5.2.3 Up-scaled Purification of Ovine Hemoglobin Pepsin Digestion Hydrolysates

The use of a semi-preparative RP-HPLC column was necessary in order to obtain sufficiently high masses of each peptide fraction for antimicrobial testing, as this column allowed for higher concentrations of sample to be loaded, whilst maintaining profile resolution.

Due to time restrictions and costs, one time point hydrolysate from each digestion condition was selected to be purified by semi-preparative RP-HPLC for antimicrobial activity determination. The time point(s) selected were to contain high yields of short antimicrobial peptides.

For the digestion of native ovine hemoglobin, the concentration of individual peptide sequences generated continually increase as more hemoglobin is digested. Therefore, in order to obtain a high yield of any given short peptide, a longer digestion period such as 24hrs would be required. Pepsin digestion of hemoglobin in the presence of urea (bovine hemoglobin at least), typically results in the generation of large intermediates first, followed by further digestion into smaller peptides. Therefore, if ovine hemoglobin were fully denatured, as expected, the digestion time point selected for purification and antimicrobial analysis would be critical as the peptide sequences existing from 0min to 24hrs vary greatly. However, because of the incomplete denaturation of ovine hemoglobin in urea resulting in few intermediates, the 24hr hydrolysate was again chosen to maximise the yield of short peptides purified by RP-HPLC.

5.2.3.1 De-salting Urea Treated Hemoglobin Hydrolysate by Gel Filtration

Before injecting the 24hr digests into the semi-preparative column, it was necessary to de-salt the 24hr urea treated hemoglobin digestion mixture by gel filtration (size exclusion chromatography). Although RP-HPLC can separate salts into fractions distinct from peptides and proteins, the presence of such a high salt concentration (urea) compared to peptide concentration meant that when trying to concentrate the peptide for loading, the salt was also concentrated. The resulting solution was too viscous for the RP-HPLC needle when at the desired protein concentration; hence a de-salting step was required.

Gel filtration separates molecules on the basis of size. The gel media consists of tiny beads containing pores of varying sizes; small molecules can enter the pores, medium-size molecules can partially enter the pores, and molecules larger than the pore size are excluded. In this way, large molecules are eluted first, followed by molecules of decreasing size, which are retarded more. A Bio-Rad P-10 gel was used to separate ovine hemoglobin and its peptides from urea present in the hydrolysate. This gel has a fractionation range of 1,500-20,000Da, meaning molecules within this size range can be separated according to their various sizes. However, for this project removing salts which are very small (urea =60.06Da) in comparison to peptides was all that was required. Therefore, any proteins

above 20,000Da were eluted first in one fraction (i.e. hemoglobin), followed by fractions of decreasing peptide sizes within the fractionation range, followed by salts.

The ability to inject a larger volume of a lower concentration would have been ideal and eliminate the need for de-salting. However the RP-HPLC machine available was analytical grade, rather than semi-preparative, with a maximum injection volume of 250ul.

5.2.3.2 Semi-preparative RP-HPLC Profiles of 24hr Hydrolysates

A new gradient (0-25% B over 15min, then 25-60% B over 35 min) was customised to suit the selected samples and the semi-preparative column, allowing for good fraction separation. Fraction separation was particularly important to ensure ease of manual collection, maximising fraction purity and providing consistency from run to run. However, due to the complexity of the hydrolysates there was expected to be a few peptides in each fraction.

Figure 5.5 shows the semi-preparative RP-HPLC chromatograms for the two 24hr ovine hemoglobin pepsin digests. It can be observed that both profiles differ from their 24hr analytical versions shown in Figure 5.2. This is most likely simply due to the diferences in column characteristics. In the case of the urea treated hydrolysate, another contributing factor to the differences observed is de-salting, where some low molecular weight peptides could have been pooled with the discarded urea fractions.

The numbered fractions in Figure 5.5 were manually collected and freeze dried before reconstitution for antimicrobial testing.

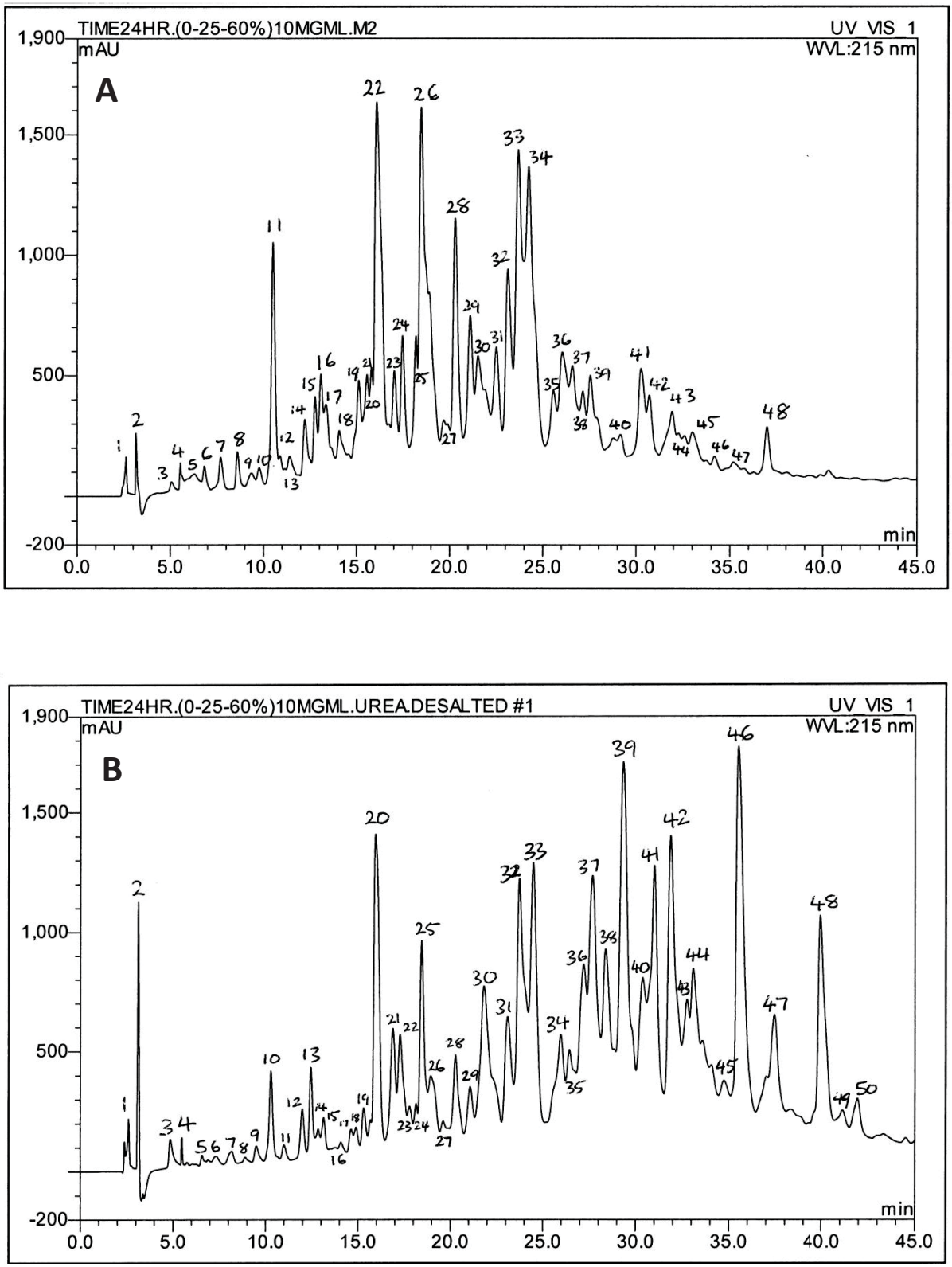


Figure 5.5 – Semi-preparative RP-HPLC chromatograms of 24hr ovine hemoglobin pepsin digestions. (A) 24hr pepsin digested native hemoglobin, (B) 24hr pepsin digested urea treated hemoglobin.

5.3 Conclusions

Ovine hemoglobin apoglobins, and peptides from hemoglobin pepsin digestion were successfully purified by semi-preparative RP-HPLC. The antimicrobial activities of these will be determined in the next chapter.

In regards to apoglobin generation, it was concluded that unlike other hemoglobin species, ovine hemoglobin subunits could not be dissociated by RP-HPLC conditions alone, at least not with the column used in this research. Therefore, acid acetone globin precipitation was required as a precursor step to RP-HPLC, where the apoglobins were fractionated.

Native ovine hemoglobin was digested by pepsin in agreement with the 'one-by-one' digestion mechanism suggested in literature. Hemoglobin molecules were digested one at a time into final peptide products, whose concentrations continually increased over the course of digestion. It was noted that hydrophobic peptides were generated first due to the preferential cleavage of hydrophobic regions by pepsin. Furthermore, due to the highly similar hydrophobicities between ovine and bovine hemoglobin, it was hypothesised based on literature results, that the N- and C- terminals of native ovine hemoglobin are likely to be digested first (due to the hydrophobicity and accessibility of pepsin to these regions), and the centre of the beta subunit last as a consequence of its relative hydrophilicity.

The digestion of ovine hemoglobin in the presence of urea did not follow the hypothesised 'zipper' mechanism. The RP-HPLC profiles described digestion kinetics that are intermediate of the 'one-by-one' and 'zipper' mechanisms. Although digestion of urea treated ovine hemoglobin was faster than the digestion of native hemoglobin, the disappearance of parental hemoglobin was much slower than characteristic of the 'zipper' mechanism, and few intermediates were formed. It was suggested that ovine hemoglobin was not fully denatured by 5.3M urea, and that dissociation of subunits may be a precursor to denaturation. Literature suggests that the ovine hemoglobin tetramer is more stable than other hemoglobin species. The use of a higher urea concentration may be adequate to dissociate and denature ovine hemoglobin or another means may be required, such as the use of strongly acidic conditions.

For the semi-preparative purification of peptides, 24hr hydrolysates were selected from both digestion conditions to maximise the yield of short peptides obtained. De-salting of the urea treated hemoglobin hydrolysate was first necessary to reduce sample viscosity in order to inject the required peptide concentration into the semi-preparative RP-HPLC column.

Chapter 6

Characterisation and Antimicrobial Activity of Ovine Hemoglobin, its Apoglobins and Peptides

6.1 Introduction

The focus of this chapter was to determine the antimicrobial activity of native ovine hemoglobin, its apoglobins and peptides derived from pepsin digestion of different structural substrates - native and denatured hemoglobin forms. It also discusses the structural features of the peptides and how they contribute to antimicrobial activity by killing microorganisms or inhibiting their growth.

In light of conflicting reports on the antimicrobial activity of native bovine and human hemoglobins *in vitro*, MICs of native ovine hemoglobin, which have not been reported until now, and those of bovine hemoglobin were determined.

Next, the activity of the alpha and beta ovine apoglobins were assessed, as it has been proposed that the separation of hemoglobin subunits exposes new antimicrobial surfaces. Supporting this are reports on the antimicrobial activities of human hemoglobin apoglobins and those from other hemoproteins, providing evidence of the enhanced activity of apoglobins in comparison to native protein tetramers.

The activity of RP-HPLC fractions from 24hr native and urea treated hemoglobin digestions were compared in terms of inhibition zones in the radial diffusion assay, and suitable candidates were selected for mass spectroscopy analysis and MIC testing.

Mass spectroscopy provides identification of the generated peptide sequences (and relative quantities), and hence the secondary structure and characteristics that influence antimicrobial activity such as hydrophobicity and net charge could be predicted. This allowed

for the observation of correlations between antimicrobial activity and the characteristics of the generated peptides, if present.

Lastly, antimicrobial activities of the ovine hemoglobin peptides in this research were compared to similar peptide sequences in the literature. It was hypothesised that the MICs of similar peptides from different hemoglobin species should exert activities within the same range, due to the highly conserved nature of the hemoglobin sequence.

6.2 Results and Discussion

Radial diffusion assays were performed to identify any antimicrobial activity exerted by native ovine hemoglobin, ovine apoglobins and the peptides from pepsin digestion. This method was selected as it is highly sensitive to antimicrobial activity and utilises minimal amounts of sample (Lehrer et al., 1991; Steinberg & Lehrer, 1997), meaning it is ideal for testing RP-HPLC fractions. The system consists of an underlay, which allows for uninhibited peptide diffusion and activity, and an overlay, which supports microbial growth.

6.2.1 Antimicrobial Activity of Native Ovine and Bovine Hemoglobin

Investigations into possible antimicrobial activity by hemoglobin have only occurred recently, as previous information suggests that native hemoglobin can bind to antibiotics, inhibit oxidative bactericidal mechanisms, and is a source of iron for microbial growth (Mak, 2008). A few research groups have more recently presented evidence that argues for and against the antimicrobial activity of native hemoglobin *in vitro*. Therefore, the activity of native ovine hemoglobin, for which there has been no known publications, and native bovine hemoglobin, for which there is conflicting evidence, were tested.

The ovine hemoglobin isolated from whole blood had to be first desalted before testing its antimicrobial activity. This is because the solution contained the anticoagulant, sodium citrate, and the lysis buffer, ammonium chloride, which are salts that may inhibit protein activity. Desalting of the ovine hemoglobin solution was carried out by gel filtration on Bio-Rad P-10 gel and the desalted sample was then freeze dried. The desalted freeze dried ovine hemoglobin sample was then reconstituted with 0.01% acetic acid to give a 30mg/ml stock, which was serially diluted down to 0.5mg/ml. 10ul of these dilutions were used in radial diffusion assays to determine the MICs of native ovine hemoglobin against common laboratory organisms.

As suggested by literature, in cases where groups have found intact hemoglobin to be antimicrobial, it is most potent against *E.coli*. For this reason *E.coli* was used as a test organism in this research, and *S.aureus* was used as a Gram positive organism, for

completeness. Radial diffusion assays were also carried out in the same way using Sigma-Aldrich powdered bovine hemoglobin to identify which claim of activity is supported for this hemoglobin species.

By following the radial diffusion assay conditions used by Parish et al. (2001), it was found that neither native ovine nor bovine hemoglobin had activity against either test organism within the concentration range tested; the MIC was >30mg/ml.

The results from this research support those of Fogaca et al. (1999) and Mak et al. (2000) (as shown in Table 6.1) who found intact human or bovine hemoglobin to possess no activity, as well as other heme-containing proteins, such as whale myoglobin and horse cytochrome c (Mak et al., 2000).

It would be expected that the results from this research would be consistent with those of Parish et al. (2001), as the same method for determination of antimicrobial activity was utilised. The results reported here are not even close to those reported by this group, where all hemoglobin species tested killed *E.coli* in micromolar concentrations. It should be noted, however, that ovine and bovine hemoglobin were not tested by this group; perhaps it is accurate to say that only certain species of hemoglobin exert antimicrobial activity. However, all mammalian hemoglobins tested by Hobson & Hirsch (1958) exerted similar activity, which makes sense due to the highly conserved sequence between different species. In agreement with the results presented in this chapter, Parish et al. (2001) did show that none of the hemoglobins tested were particularly effective against *S.aureus*, as the MIC was 20mg/ml for three of the hemoglobin species against this organism.

Table 6.1 - Antimicrobial activities of native hemoglobin species complied from literature.

Hemoglobin Species	Test Organism	Measure of Activity	Activity	Author
Ovine	*E. coli* 0111	MIC - radial diffusion assay (mg/ml)	No activity[a]	This research
Ovine	*S. aureus* NCTC 4163		No activity[a]	
Bovine	*E. coil* 0111		No activity[a]	
Bovine	*S. auerus* NCTC 4163		No activity[a]	
Alligator	*E. coli* K12	MIC (uM)	25	Parish et al. (2001)
Alligator	*S. faecalis*		ND*	
Alligator	*S. aureus*		ND*	
Alligator	*C. albicans*		20000	
Human	*E. coli* K12		2	
Human	*S. faecalis*		1500	
Human	*S. aureus*		20000	
Human	*C. albicans*		50	
Horse	*E. coli* K12		2	
Horse	*S. faecalis*		20000	
Horse	*S. aureus*		20000	
Horse	*C. albicans*		250	
Snake	*E. coli* K12		10	
Snake	*S. faecalis*		15	
Snake	*S. aureus*		20000	
Snake	*C. albicans*		100	
Human	*E. coli*	LD_{50} (half maximal lethal dose) (uM)	No activity[b]	Mak et al. (2000)
Human	*Salmonella*		No activity[b]	
Human	*P. aeruginosa*		No activity[b]	
Human	*K. oxytoca*		No activity[b]	
Human	*E. faecalis*		No activity[b]	
Human	*S. aureus*		No activity[b]	
Human	*C. albicans*		No activity[b]	
Bovine	*M. luteus*	MIC (uM)	No activity[c]	Fogaca et al. (1999)
Human	*E. coli* K12	Minimum concentration of hemoglobin for 50% killing (ug/ml)	0.02	Hobson & Hirsch (1958)
Bovine	*E. coli* K12		0.1	
Rabbit	*E. coli* K12		0.02	
Mouse	*E. coli* K12		0.1	
Rat	*E. coli* K12		0.02	
Guinea pig	*E. coli* K12		0.02	

No activity at highest test concentration of 30mg/ml[a], 330uM[b] or 30uM[c], *MIC not determined by author and the reason was not stated.

It is likely that the differences between the results presented here and those in the literature are dependent on the conditions of the antimicrobial testing method and/or possible contamination. All of the groups mentioned in Table 6.1 isolated hemoglobin from whole blood; it is a possibility that in cases where activity was identified, other antimicrobial compounds from blood were present in the hemoglobin samples assayed. In the case of the research in this thesis, small amounts of serum albumin were present in the hemoglobin sample, as identified by mass spectrometry data. This is potentially antagonistic to antimicrobial activity, although small amounts of BSA are commonly used in microtitre broth assays to prevent peptide binding to containers (Steinberg & Lehrer, 1997). A more likely explanation for the differences in results are the test conditions used, including variations in microorganism strains and assay parameters such as ionic strength and pH.

After further investigation into assay parameters, it was thought that pH may be the critical factor explaining the widely reported differences in antimicrobial activities of hemoglobin, as a reduction in pH reduces the alpha helical content of hemoglobin. Hobson & Hirsch (1958) found that hemoglobin antimicrobial activity was abolished at pH 6 and above. This group therefore carried out their testing at pH 5.0, and found various hemoglobin species to be active against *E.coli* K12. Parish et al. (2001) used a pH of 5.5 and got potent activities against *E.coli* also, whereas Mak et al. (2000) tested activities in Hanks balanced salt solution (HBSS), which has a physiological pH and salt content, and obtained no activity. All other aspects of the testing methods that each group utilised appeared to promote antimicrobial activity i.e. low ionic concentration, the use of mid-log cultures and sufficient incubation time at 37/38^{o}C.

Native ovine and bovine hemoglobin activities were retested using the same radial diffusion assay method, but at a lower pH of 4.5. Still no activity was observed, so it was concluded in agreement with Mak et al. (2000), that intact hemoglobin does not exert antimicrobial activity.

The groups who found hemoglobin to be antimicrobial did state that it is unlikely to be active *in vivo* as the conditions under which it is active *in vitro* are low ionic strength and acidic pH, which are not reflective of conditions in most parts of the body.

6.2.2 Antimicrobial Activity of Apoglobins from Ovine Hemoglobin

Although antimicrobial activity of the hemoglobin tetramer has not been agreed upon, groups that have investigated the activity of its subunits and apoglobins have provided evidence supporting antimicrobial activity of these monomers.

The two freeze dried RP-HPLC fractions of acid acetone precipitated ovine apoglobins were reconstituted in 0.01% acetic acid to give a 4mg/ml stock, which was serially diluted down to 4ug/ml. 10ul of each concentration was assayed against the laboratory organisms, *E.coli*, *S.aureus* and *C.albicans*. The following Table 6.2 shows the activities of each apoglobin against the different test organisms.

Table 6.2 - MIC values of acid acetone precipitated apoglobins from ovine hemoglobin.

RP-HPLC Fraction	MIC Against Test Organism (mg/ml)		
	E.coli	*S.aureus*	*C.albicans*
1	0.5-1.0	0.5-1.0	No activity*
2	1.0-2.0	0.5-1.0	No activity*

*No activity at the highest test concentration of 4.0mg/ml.
See Figure 5.1 for apoglobin RP-HPLC profile showing fractions 1 and 2.
MIC tests were carried out in duplicate for each test organism and fraction. The results from duplicates were identical as the radial diffusion assay was not sensitive enough to differentiate MIC values within the upper ranges of the serial dilution concentrations i.e. any MIC that fell between 0.5 and 1.0mg/ml was taken as 1.0mg/ml, as zone inhibition was present at 1.0mg/ml but not at 0.5mg/ml. Intermediate concentrations could be tested if more of each fraction was available.

These results show that both apoglobins from ovine hemoglobin exerted moderate activity against *E.coli* and *S.aureus*. However, neither apoglobin was active towards the fungi, *C.albicans*, at the highest assay concentration of 4 mg/ml. Literature MIC values for globins against *C.albicans*, presented in Table 6.3 are also higher in comparison to other test organisms. This is likely due to the different cell wall structure that fungi possess, which affects the ability of peptides to permeate it. MIC values reported by Mak et al. (2000) also show that human alpha and beta apoglobins exert activity within the same range of each other. However, results by Parish et al. (2001) suggest that the beta globin is much less active than the alpha form. Again, there is a huge difference in the magnitude of MIC values

presented by each group. As mentioned earlier, these variations likely represent differences in test conditions and the measures of activity.

Table 6.3 - Antimicrobial activity of hemoglobin apoglobins and subunits compiled from literature.

Apoglobin or Subunit from Hemoglobin	Test Organism	Measure of Activity	Activity	Author
Ovine apoglobin (fraction 1)	*E.coli* 0111	MIC (mg/ml)	0.5-1.0	This research
Ovine apoglobin (fraction 2)	*E.coli* 0111		1.0-2.0	
Ovine apoglobin (fraction 1)	*S.aureus* NCTC 4163		0.5-1.0	
Ovine apoglobin (fraction 2)	*S.aureus* NCTC 4163		0.5-1.0	
Ovine apoglobin (fraction 1)	*C.albicans* 3153A		No activity[a]	
Ovine apoglobin (fraction 2)	*C.albicans* 3153A		No activity[a]	
Human (alpha apoglobin)	*E.coli*	MIC (uM)	2	Parish et al. (2001)
Human (alpha subunit)	*E.coli*		1	
Human (beta apoglobin)	*E.coli*		4	
Human (beta subunit)	*E.coli*		3	
Human (alpha apoglobin)	*S. faecalis*		3	
Human (alpha subunit)	*S. faecalis*		2	
Human (beta apoglobin)	*S. faecalis*		20	
Human (beta subunit)	*S. faecalis*		15	
Human (alpha apoglobin)	*S. aureus*		15	
Human (alpha subunit)	*S. aureus*		ND*	
Human (beta apoglobin)	*S. aureus*		200	
Human (beta subunit)	*S. aureus*		ND*	
Human (alpha apoglobin)	*C.albicans*		7	
Human (alpha subunit)	*C.albicans*		15	
Human (beta apoglobin)	*C.albicans*		300	
Human (beta subunit)	*C.albicans*		10000	
Human (alpha apoglobin)	*E.coli*	LD_{50} (half maximal lethal dose) (uM)	3.6	Mak et al. (2000)
Human (beta apoglobin)	*E.coli*		4.2	
Human (alpha apoglobin)	*Salmonella*		5.1	

Apoglobin or Subunit from Hemoglobin	Test Organism	Measure of Activity	Activity	Author
Human (beta apoglobin)	*Salmonella*	LD_{50} (half maximal lethal dose) (uM)	4.4	Mak et al. (2000)
Human (alpha apoglobin)	*P. aeruginosa*		4.4	
Human (beta apoglobin)	*P. aeruginosa*		6.7	
Human (alpha apoglobin)	*K. oxytoca*		5.8	
Human (beta apoglobin)	*K. oxytoca*		8.3	
Human (alpha apoglobin)	*E. faecalis*		6.5	
Human (beta apoglobin)	*E. faecalis*		8.0	
Human (alpha apoglobin)	*S. aureus*		7.3	
Human (beta apoglobin)	*S. aureus*		7.1	
Human (alpha apoglobin)	*C. albicans*		14.0	
Human (beta apoglobin)	*C. albicans*		7.5	
Horse (alpha and beta apoglobins)	*E.coli* K12	Minimum concentration of hemoglobin for 50% killing (ug/ml)	0.4	Hobson & Hirsch (1958)

[a]No activity at highest test concentration of 4mg/ml, *MIC not determined by author and the reason was not stated.

The findings of the research presented here do support those of the literature in the sense that apoglobins were found to be many times more active against microorganisms than intact hemoglobin, which may not be active at all, as found in this research. This has also been found to be true for apoglobins from other heme-containing proteins, such as myoglobin and cytochrome c (Mak et al., 2000).

The lack of activity of native hemoglobin in comparison to its apoglobins may be due to a couple of reasons. By analysing the information presented by Parish et al. (2001), it appears that the presence of heme is not necessary for antimicrobial activity; globins with or without heme exert antimicrobial activity. In fact, in some cases the removal of heme dramatically enhances activity. A more likely explanation is that the separation of subunits exposes new antimicrobial surfaces (Parish et al., 2001), which are normally hidden within the hemoglobin

tetramer structure. Mak et al. (2008 & 2000) concluded that for a hemoprotein to be antimicrobial, it must be deprived of heme and partially unfolded. The research presented here supports this.

6.2.3 Antimicrobial Activity of Peptides from Pepsin Digestion of Ovine Hemoglobin

6.2.3.1 Zone Inhibition of *E.coli* 0111 by Ovine Hemoglobin 24hr Pepsin Digestion Fractions

Semi-preparative RP-HPLC fractions of the 24hr ovine hemoglobin pepsin digestions were tested by radial diffusion assay to identify which fractions had antimicrobial activity.

The freeze dried fractions were reconstituted in very small volumes (15ul) of 0.01% acetic acid to give concentrated solutions for assaying. This volume allowed for 10ul to be used in the activity assay and the remainder for mass spectrometry analysis. Fractions were first tested against *E.coli* as this appears to be one of the most sensitive organisms to antimicrobial peptides.

The following Table 6.4 shows the resulting inhibition zone diameters of each RP-HPLC fraction against *E.coli* 0111. These fractions correspond to those presented in Figure 5.5. Diameters were calculated by subtracting the well size (4.4mm) from the clearing diameter determined by a microcaliper.

Table 6.4 – Inhibition diameters of RP-HPLC fractions from 24hr ovine hemoglobin pepsin digestion hydrolysates against E.coli 0111.

Fraction Number	Inhibition Zone Diameter (mm)	
	Native Hemoglobin Digest[a]	Urea Treated Hemoglobin Digest[b]
16	0.2	-
17	-	-
18	-	2.1
19	1.4	-
20	1.2	6.4
21	-	-
22	2.2	3.4
23	-	-
24	0.2	1.7
25	-	-
26	2.1	-
27	-	-
28	3.0	-
29	-	-
30	1.2	4.7
31	0.9	2.6
32	2.7	-
33	0.1	2.3
34	0.8	0.9
35	-	2.7
36	1.5	2.4
37	-	2.1
38	-	2.7
39	1.4	4.5
40	-	0.8
41	0.5	2.7
42	-	6.7
43	0.3	2.3
44	0.4	2.0
45	0.3	2.6
46	-	7.1
47	0.3	4.9
48	0.3	2.8
49	NR*	0.3
50	NR*	2.3

'-' indicates no activity was observed (whole RP-HPLC fractions dissolved in 15ul of 0.01% acetic acid and 10ul assayed), *Not relevant due to absence of this fraction, as seen in Figure 5.5(A).
See Figure 5.5(A)[a] and 5.5(B)[b] for RP-HPLC profiles showing peptide fractions.
Note: the inhibition zones of fractions between the two digestion conditions are not directly comparable i.e. fraction 20 from the native hemoglobin digest does not correspond to fraction 20 of the urea treated hemoglobin digest.

Fractions earlier than fraction 16 in the native hemoglobin digest, and fraction 18 in the urea treated hemoglobin digest did not have activity against *E.coli* 0111, so were not included in Table 6.4. These correspond to retention times of less than 13min and 15min respectively. This lack of activity could be due to the greater hydrophilicity of peptides that elute at earlier time points and/or due to the lower concentration of these fractions, as seen in the RP-HPLC profiles in chapter 5, Figure 5.5. Fraction 11 in the native hemoglobin digest (Figure 5.5(A)) may be a good example of hydrophilicity having an effect on antimicrobial activity; it has almost the same peptide concentration as fraction 28 (a more hydrophobic fraction). Yet fraction 11 showed no activity towards *E.coli*, whereas fraction 28 had the largest inhibition zone of any fraction in that digestion condition. Therefore, this suggests that the peptides within these two fractions possess different characteristics, which result in different levels of success in permeating the *E.coli* membrane. Moreover, a fraction with a low peptide concentration can have as much activity as a very concentrated one if the peptides in the first are a lot more active in comparison to the second.

It can be observed that the inhibition zones from the urea treated hemoglobin digest fractions are significantly larger than those from the native hemoglobin digest, and more of these fractions are antimicrobial. Again, this could be due to a higher concentration of peptides in the urea digest fractions, and/or the peptide sequences generated under this condition could be more active than those from the native digest.

On the basis of significantly larger inhibition zone diameters, RP-HPLC fractions from the urea treated hemoglobin digest were chosen for further testing against different test organisms. From this, fraction candidates were selected for mass spectrometry and MIC testing.

6.2.3.2 Zone Inhibitions by Urea Treated Ovine Hemoglobin 24hr Pepsin Digest Fractions

Table 6.5 compares the activity of the RP-HPLC fractions from the 24hr urea treated hemoglobin digest against three test organisms. A Gram negative bacteria, a Gram positive bacteria, and a fungus were chosen to test for broad spectrum activity.

Table 6.5 - Inhibition zones of RP-HPLC fractions from the 24hr pepsin digestion of urea treated ovine hemoglobin against different test organisms.

Fraction Number	Inhibition Zone Diameters		
	E.coli 0111[a] (mm)	*S.aureus*[b] (mm)	*C. albicans*[c] (mm)
18	2.1	-	-
19	-	-	-
20	6.4	2.0	-
21	-	-	-
22	3.4	2.7	-
23	-	-	-
24	1.7	-	-
25	-	-	-
26	-	-	-
27	-	-	-
28	-	-	-
29	-	-	-
30	4.7	NR*	-
30.1	NR*	-	-
30.2	NR*	2.6	-
31	2.6	3.1	-
32	-	2.3	-
33	2.3	6.2	-
34	0.9	2.1	-
35	2.7	-	-
35.1	NR*	-	-
36	2.4	NR*	-
36/37	NR*	7.7	-
37	2.1	NR*	-
38	2.7	2.1	1.5
39	4.5	4.2	-
40	0.8	2.5	-
41	2.7	-	-
42	6.7	3.7	-
43	2.3	-	-
44	2.0	2.9	-
45	2.6	-	-
46	7.1	3.2	-
47	4.9	-	-
48	2.8	5.2	-
49	0.3	-	-
50	2.3	-	-

'-' indicates no activity was observed (whole RP-HPLC fractions dissolved in 15ul of 0.01% acetic acid and 10ul assayed), *Not relevant due to the variability in RP-HPLC profile resolution between separate RP-HPLC runs. See Figure 5.5(B)[a] and Appendix 2.1[b,c] for RP-HPLC profiles corresponding to each zone inhibition assay.

Table 6.5 shows that more fractions are active against *E.coli* 0111 than *S.aureus* or *C.albicans*, with only one fraction (fraction 38) exerting activity against *C.albicans*. Fractions that are active towards both *E.coli* and *S.aureus* are generally much more potent against one or the other, and this distribution appears to be approximately even between the two organisms.

Due to time and cost restrictions only six fractions could be selected for mass spectroscopy analysis. Fractions that met the selection criteria had significant antimicrobial activities against more than one test organism (i.e. are broad spectrum) and were distinct from other fractions on the RP-HPLC chromatograms. The collection of well resolved peaks means that there is less potential for contamination from other fractions, providing accurate mass spectrometry data and runs than have consistent composition between MIC assays.

By comparing inhibition zone data with peak characteristics from RP-HPLC profiles, fractions 20, 38, 39, 42, 46, and 48 were selected for mass spectrometry analysis. Fraction 38 was included in this group because although it gave smaller inhibition zones, it was the only fraction active against all three test organisms.

6.2.3.3 Ovine Hemoglobin Peptide Identification by Mass Spectrometry

Matrix-assisted laser desorption imaging (MALDI) mass spectrometry was carried out on the six RP-HPLC fractions from the 24hr urea treated hemoglobin pepsin digest, as described in the methods section. The outputs were the following spectra (Figures 6.1 to 6.6), showing the relative current intensity, which corresponds to the relative abundance of each peptide (in ion form), versus their mass to charge ratio (m/z).

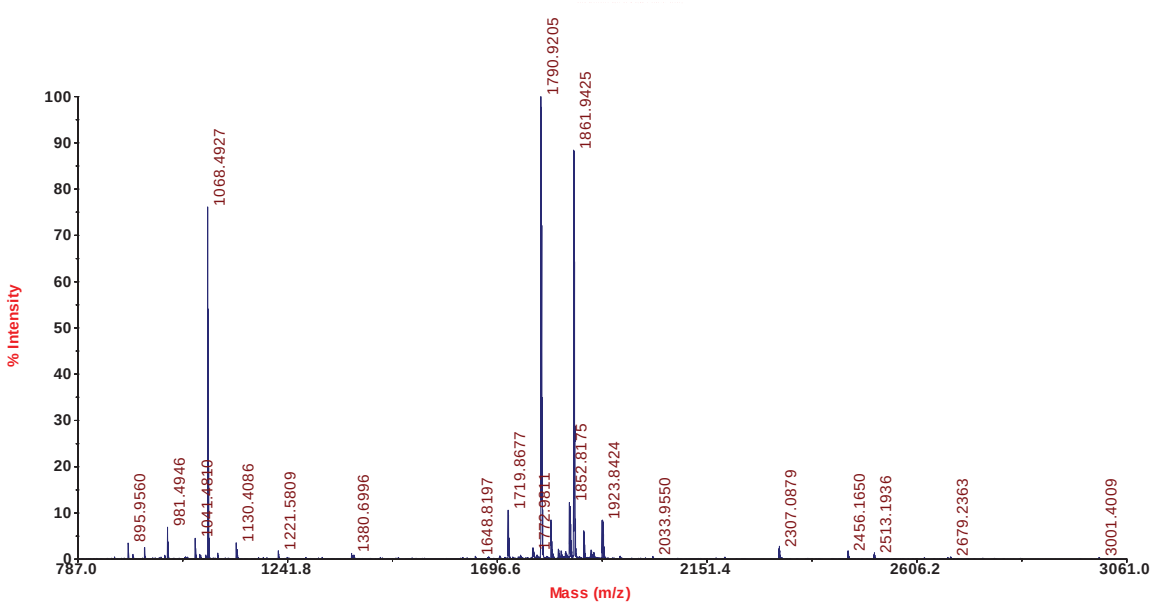


Figure 6.1 - Fraction 20 mass spectrometry image.

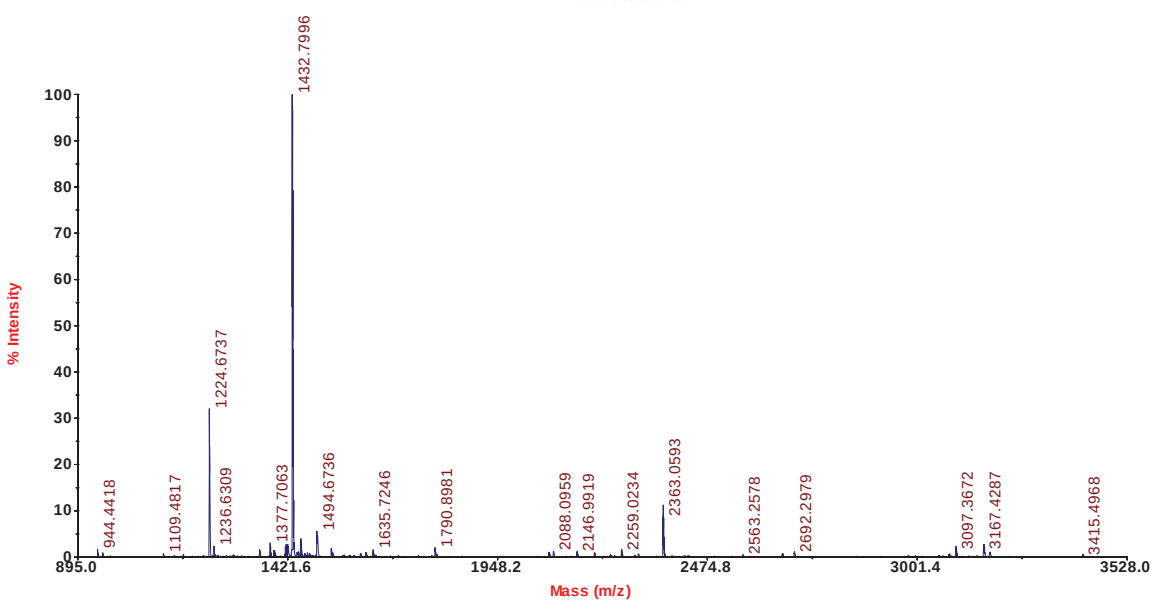


Figure 6.2 - Fraction 38 mass spectrometry image.

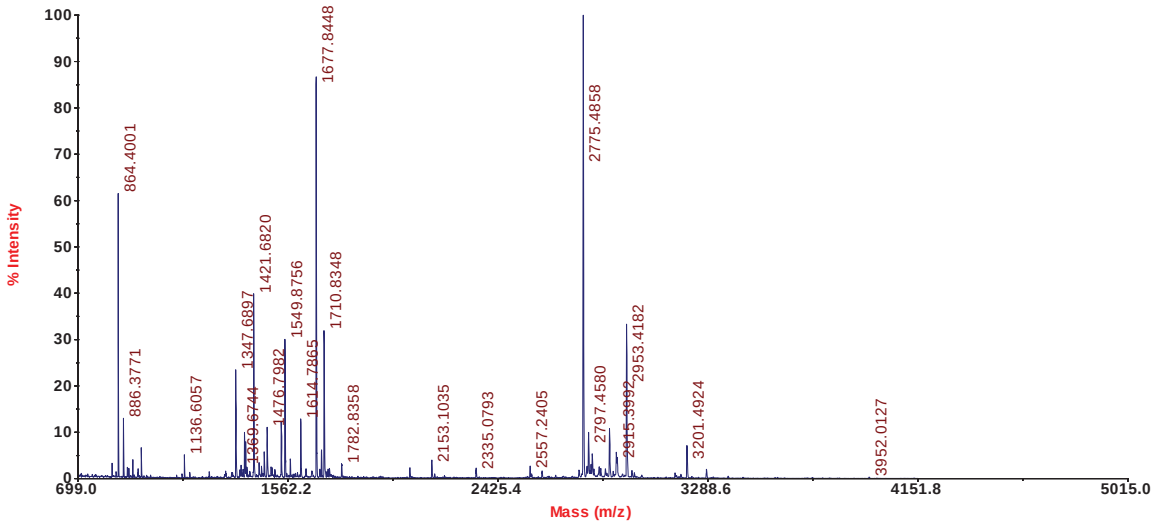


Figure 6.3 - Fraction 39 mass spectrometry image.

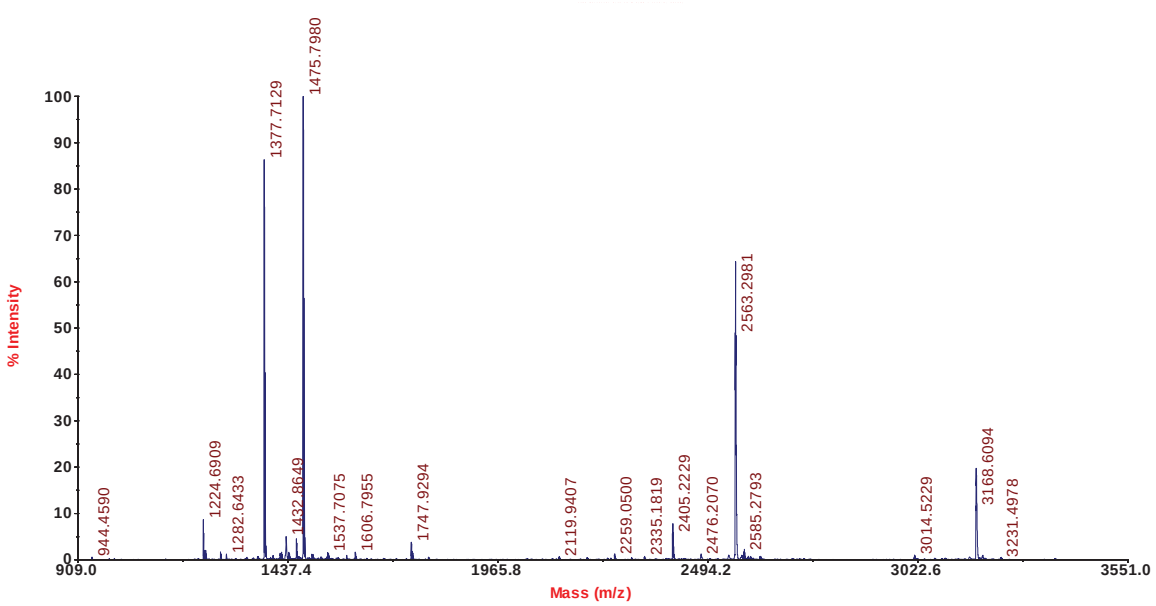


Figure 6.4 - Fraction 42 mass spectrometry image.

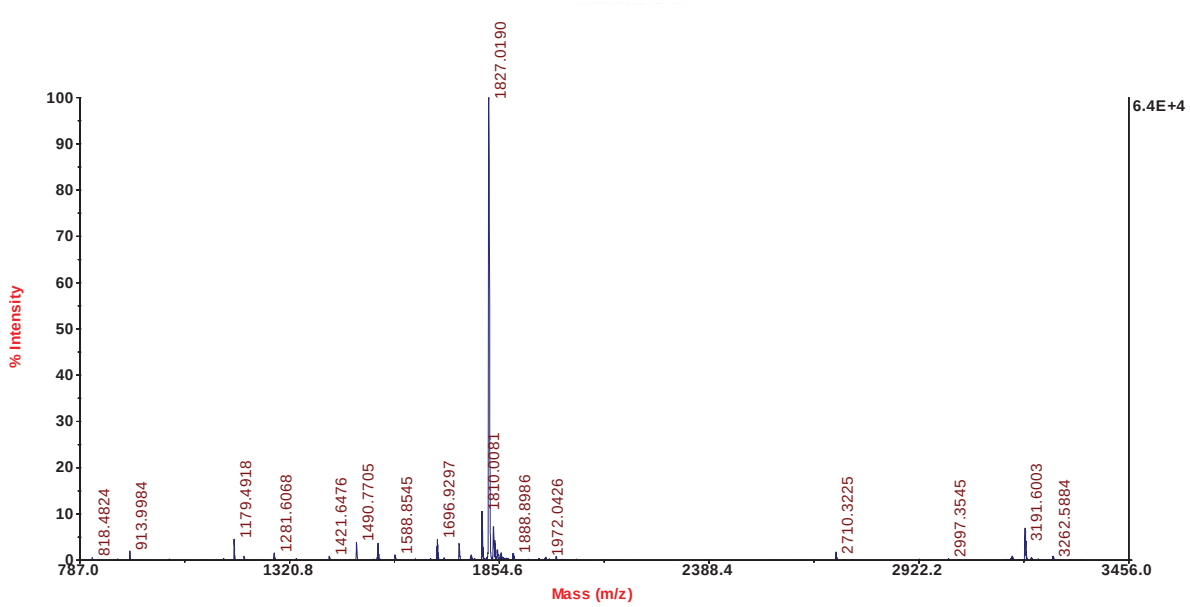


Figure 6.5 - Fraction 46 mass spectrometry image.

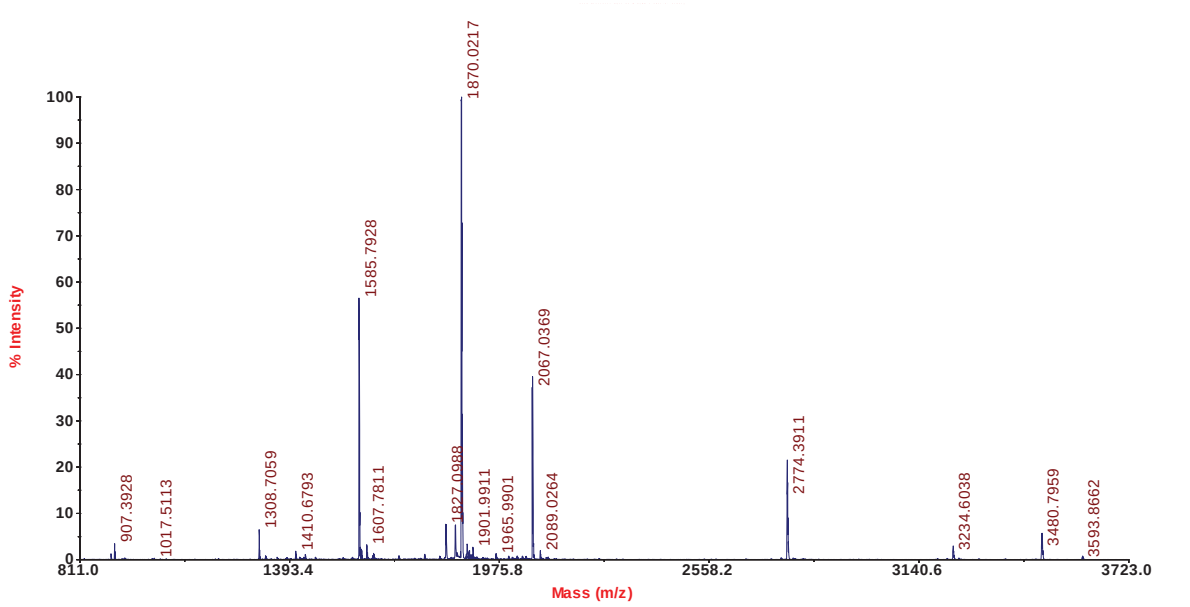


Figure 6.6 - Fraction 48 mass spectrometry image.

To identify peptide sequences, a MS/MS ion search was carried out comparing the MS data of the samples against the UniProt/SWISS-PROT amino acid data base, using the Mascot search engine. Search parameters were set up to include some possible modifications, such as deamidation (D, N), oxidised methionine and pyroglutamate (E, Q), and the enzyme field was set for no enzyme because pepsin is a non-specific protease. Table 6.6 gives the identities of the peptides in each fraction.

Table 6.6 - Identification of ovine hemoglobin peptides by mass spectrometry.

Fraction Number	Molecular Mass (Da)	Sequence	Origin
20	1790.9080	-DLSHGSAQVKGHGEKVAA-	48-65 alpha chain
	1861.9325	-DLSHGSAQVKGHGEKVAAA-	48-66 alpha chain
	1068.4862	-ARHHGNEFT-	114-122 beta chain (allele B)
38	1432.8132	-HAHKLRVDPVNF-	88-99 alpha chain
	1224.6737	-HKLRVDPVNF-	90-99 alpha chain
39	1475.7980	Unidentified	Unidentified
	1377.6920	-ARHHGNEFTPVL-	114-125 beta chain (allele B)
	2563.2476	-SAADKSNVKAAWGKVGGNAGAYGAEAL-	4-30 alpha chain
	3168.5388	-FEHFGDLSNADAVMNNPKVKAHGKKVLDS-	41-69 beta chain (allele B)
42	2775.4858	-VLSAADKSNVKAAWGKVGGNAGAYGAEAL-	2-30 alpha chain
	1677.8445	-FSNGVQHLDDLKGTF-	67-81 beta-C chain
	864.4001	-FEHFGDL-	41-47 beta chain (allele A or B)
46	1827.0189	-WGKVKVDEVGAEALGRL-	14-30 beta chain (allele A or B)
48	1870.0217	Unidentified	Unidentified
	1585.8052	-LSFPTTKTYFPHF-	35-47 alpha chain
	2067.0618	-TKAVGHLDDLPGTLSDLSDL-	68-87 alpha chain
	2774.4358	-DDLPGTLSDLSDLHAHKLRVDPVNF-	75-99 alpha chain

Major peptides within each fraction are listed from most abundant to least abundant.

It is noticeable that the peptides in the fractions analysed fulfil the criteria of being short, meaning they are more likely to lack secondary structure (random coiled peptides), which is the desired outcome for this research. They have a molecular mass range of 864-3168Da, and are between 7-29 amino acids long.

6.2.3.4 Origins of Major Peptide Products from Urea Treated Ovine Hemoglobin Pepsin Digestion

Figure 6.7 shows the origin of each of the major fragments in the six RP-HPLC fractions analysed. These correspond to the peptides listed in Table 6.6.

Beta subunit: allele A

1 mltaeekaav tgf**wgkvkvd evgaealgrl** lvvypwtqrf **fehfgdl**ssa davmnnakvk

61 ahgkkvldsf sngvqhlddl kgtfaqlsel hcdklhvdpe nfrllgnvlv vvlarhhgse

121 ftpvlqaefq kvvagvanal ahryh

Beta subunit: allele B

1 mltaeekaav tgf**wgkvkvd evgaealgrl** lvvypwtqrf **fehfgdlsna davmnnpkvk**

61 **ahgkkvlds**f sngmkhlddl kgtfaqlsel hcdklhvdpe nfrllgnvlv vvl**arhhgne**

121 **ftpvl**qadfq kvvagvanal ahkyh

Beta subunit: allele C

1 mpnkalitgf wskvkvdevg aealgrllvv ypwtqrffeh fgdlstadav lgnakvkahg

61 kkvlds**fsng vqhlddlkgt f**aqlselhcd klhvdpenfr llgnvlvvvl arhfgkeftp

121 elqaefqkvv agvasalahr yh

Alpha subunit

1 m**vlsaadksn vkaawgkvgg nagaygaeal** ermf**lsfptt ktyfphfdls hgsaqvkghg**

61 **ekvaaa**l**tka vghlddlpgt lsdlsdlhah klrvdpvnf**k llshsllvtl achlpndftp

121 avhasldkfl anvstvltsk yr

Figure 6.7 - Origin of major peptide products within ovine hemoglobin subunits. Bold coloured writing corresponds to major fragments identified by mass spectrometry. Note that beta alleles A and B are adult forms, whilst allele C is the foetal form. Arrows show the sequences where the major fragments described in Table 6.6. were obtained.

From the six fractions analysed, it is clear that a large number of the identified peptides originate from the alpha subunit, and to a lesser extent the beta subunit allele B. However, since these fractions are only a proportion of the total hydrolysate it cannot be said if this is representative of the entire digestion sample. The relative percentages of ovine hemoglobin alpha and beta subunits digested by pepsin could only be known by obtaining mass spectrometry data on all generated fragments. However, this result is consistent with the pepsin digestion of bovine hemoglobin (both native and urea treated) by Su et al. (2007a, 2007b), where they found approximately twice as many peptides were generated from the alpha subunit as the beta subunit. Furthermore, the presence of more peptides originating from the beta subunit allele B as opposed to allele A is likely to simply reflect blood sampling from a sheep population largely homozygous for hemoglobin B.

It can be seen that in most cases pepsin did cleave at the expected positions, which is at the C-terminal of residues containing bulky groups i.e. phenylalanine (F) and leucine (L). This cleavage pattern is represented in Figure 6.7 by the black arrows.

6.2.3.4.1 Peptides Unidentified by Mass Spectrometry

Table 6.6 shows that two of the most abundant peptides in fraction 39 and 48 were unidentified. Identification of proteins/peptides is largely dependent on MS data quality, database accuracy and its completeness, and inputs/search parameters into the search engine. However, when this information is of a high standard and peptides remain unidentified, there are a few possibilities for this. In the case of peptides present in low abundance, too little fragment ion in the spectrum (Flikka et al., 2006) is a common reason for lack of identification. This was observed for a few of the very minor peptide products in this research (data not shown). However, in the case of the two major unidentified products, the main reasons for lack of identification are sample contamination where the spectra is not a peptide, or the peptide has undergone a modification that is unaccounted for by the database (Flikka et al., 2006). Modifications may be natural or artificial i.e. they are post-translational or are caused by chemicals in the sample preparation and processing (Lubec & Afjehi-Sadat, 2007). However, it is said that chemical modifications account for a significant proportion of unidentified structures (Lubec & Afjehi-Sadat, 2007).

In this research, precautions were always taken to minimise contamination, and the search parameters accounted for some of the most common post-translational modifications. Therefore, it is most likely that the unidentified peptides are the result of chemical modification. For example, Mak et al. (2000) states that when digesting human hemoglobin with CNBr some peptides have N-terminal methionine converted to homoserine lactone, and when digested with Arg-C, peptides can have cysteines converted to carbamidomethylo-cysteine. However, there are hundreds of natural and artificial modification possibilities, which make it extremely difficult to reach a conclusion about the peptide identities without doing further testing.

6.2.3.5 MIC Assays of Selected Ovine Hemoglobin Peptide Fractions from Pepsin Digestion

The MICs of the six ovine hemoglobin peptide fractions with mass spectrometry identities were to be confirmed. As a starting point, the RP-HPLC peptide standard curve (see Appendix 2.2) was used to calculate the peptide concentration of each fraction that was required to produce the inhibition zones in Table 6.5. The concentration of peptide in these fractions was determined to be very high, so it was assumed that activity would be retained after a significant dilution, as most active AMPs exert activity in microgram per millilitre concentrations. Each freeze dried fraction was reconstituted in 100ul of 0.01% acetic acid and successive dilutions from this were made for MIC determination by radial diffusion assay, pH 5.5. However, it was found that even at the highest concentration tested, no growth inhibition occurred.

The assay was then repeated using higher peptide concentrations i.e. concentrations in the range of those that initially produced zone clearing. This required fractions from three RP-HPLC runs to be pooled together to give enough material for MIC testing; the exact concentration of these fractions were again determined by the standard curve. From this, dilutions were made for MIC testing and 10ul of each was assayed.

It was determined that the MICs of all six fractions were very high against all three test organisms. Consistent with results from Table 6.5, only fraction 38 exerted activity towards

C.albicans at the highest test concentration, and this was weak activity also. Table 6.7 shows the MIC of each fraction against the three test organisms.

Table 6.7 - MICs of pepsin digested ovine hemoglobin RP-HPLC fractions.

Fraction number	MIC range (mg/ml)		
	E.coli	*S.aureus*	*C.albicans*
20	16-20	25-31	No activity[a]
38	15-18	12-15	13-16
39	30-37	33-41	No activity[b]
42	22-28	23-29	No activity[c]
46	29-36	35-44	No activity[d]
48	17-21	22-28	No activity[e]

No activity at highest test concentration of 31mg/ml[a], 43mg/ml[b], 30mg/ml[c], 46mg/ml[d] or 33mg/ml[e].
Single radial diffusion assays were carried out as each assay required pooling three independent RP-HPLC runs to allow for visible zone inhibition. Ideally these assays would be replicated. However, their purpose here was to screen for potential potent AMPs, which can later be synthesized in larger quantities and tested, rather than testing for highly accurate MIC values outright.
Underlay was set at pH 5.5 and fractions were dissolved in 0.01% acetic acid.
Various pH (4.5. 5.5, and 7.4) and reconstitution agents (sodium phosphate, acetic acid, water) were implemented but offered no improvement to MIC values.

6.2.3.5.1 MICs of Synthetic Ovine Hemoglobin Peptides

In this project, a few synthetic ovine hemoglobin peptides were made for antimicrobial testing also, based on literature evidence that suggests strong MICs of these peptides:

- beta-A(140-145) -LAHRYH-
- beta-B(140-145) -LAHKYH-
- beta-B(129-145) -FQKVVAGVANALAHKYH-
- alpha(96-107) -PVNFKLLSHSLL-

In particular, the sequence LAHRYH from bovine hemoglobin, which is conserved in the ovine hemoglobin beta subunit allele A, has been reported as highly active against *E.coli, S.enteritidis, L.innocua, and M.luteus*, with MIC values ranging from 2 to 10uM (Nedjar-Arroume et al., 2008). Also, -FQKVVAGVANALAHKYH- falls within a widely reported antimicrobial region from the hemoglobin beta subunit. This region is highly conserved between hemoglobin species. These peptides were tested for their MICs against *E.coli* and

S.aureus, using the same radial diffusion assay at pH5.5 which is used elsewhere in this research. However, only beta-B(129-145) was found to be active at the highest test concentration of 2mg/ml, and this was only against *E.coli*.

Due to these unexpectedly poor MIC results, another common method for determining antimicrobial activity was performed - the microtitre broth assay. In this method the peptides were serially diluted two-fold in 0.01% acetic acid plus 0.2% BSA, and log-phase cells were added and incubated overnight. The last well with clearing, i.e. no growth, corresponds to the MIC value. However, none of the peptides tested in this research (aside from positive controls) were active under the conditions of this system. This may be due to the presence of BSA, which is a serum protein that may inhibit peptide activity. Nedjar-Arroume et al. (2008) were contacted several times in regards to their MIC values from pepsin digestion of bovine hemoglobin, without response.

It was concluded that none of the ovine hemoglobin digest fractions or synthetic peptides are particularly active against *E.coli, S.aureus or C.albicans* - only in milligram per millilitre concentrations. To assess why, the structural characteristics of the peptides in each fraction and each synthetic peptide were examined.

Interestingly, towards the end of this research a conference abstract, 'An Ovine Hemoglobin Fragment Inhibits *Porphyromonas gingivalis* and *Micromonas micros*' by Steward-Tharp et al. (2007) was discovered, and the group was contacted for further information (Brogden, 2011). The group found a 36 amino acid peptide from the C-terminus of the beta chain of sheep hemoglobin (in tracheal lysates) to be active against *E.coli*. However, when synthesised, the peptide showed no activity against *E.coli, S.aureus, P.aeruginosa, S.marcescens or A.actinomycetemcomitans*. Unusually, the peptide was active in microgram per millilitre concentrations against two oral bacteria, which are typically resistant to defensins and cathelicidins. The mean MIC values were 250ug/ml and 99ug/ml against *M.micros* and *P.gingivalis*, respectively. No further work was published on this, but perhaps the peptides generated in the research presented in this thesis are also more active towards a different group of microorganisms. Furthermore, it is interesting to note the lack of activity of the synthetic form against *E.coli*, suggesting that a modification is likely necessary for

antimicrobial activity of this peptide. This may also be the case for the peptides synthesised here.

In terms of antimicrobial activity, peptide modifications can enhance, diminish or have no effect (Andreu & Rivas, 1998). C-terminal amidation has been found to be essential for the potent activity of certain ovine AMPs, such as SMAP29, OaBac5mini and OaBac7.5 (Anderson et al., 2004). Furthermore, studies have been carried out looking at the activity of the amidated and non-amidated versions of bovine hemoglobin alpha(33-61), the first identified antimicrobial hemoglobin peptide, which was found in the tick gut (Mak, 2008). It was found that the C-terminally amidated form had increased activity against Gram positive bacteria and four times the activity against *C.albicans* compared to the non-amidated form (Machado et al., 2007). The increase in peptide activity due to amidation is thought to be attributed to enhanced resistance to peptide proteolysis (Mak, 2008; Sforca et al., 2005), or a conformational change (Machado et al., 2007).

6.2.3.6 Contribution of Peptide Structural Characteristics to Antimicrobial Activity

It can be seen from the mass spectra in Figures 6.1-6.6 that some fractions are more complex than others, making it difficult to identify what component(s) contribute to the observed antimicrobial activity. Therefore, the structural features of the identified peptides were compared to structural features that are typically known to confer antimicrobial activity in order to speculate why these fractions have low activity. This information can be seen in Table 6.8. Of course, it is not possible to determine this information on the two unidentified peptides or their contribution to antimicrobial activity. Their abundance does not mean that they are highly active or are active peptides at all.

Table 6.8 – Properties of peptides generated by pepsin digestion of ovine hemoglobin, and synthetic ovine hemoglobin peptides.

Fraction Number	Sequence	Number of Amino Acids	% Hydrophobic Residues	Charge at pH 7, 5.5	Secondary Structure Prediction
20	-DLSHGSAQVKGHGEKVAA-	18	33	0, +2	89% random coil
	-DLSHGSAQVKGHGEKVAAA-	19	37	0, +2	10.5% alpha helix, 74% random coil
	-ARHHGNEFT-	9	22	0, +2	100% random coil
38	-HAHKLRVDPVNF-	12	42	+1, +3	100% random coil
	-HKLRVDPVNF-	10	42	+1, +2	100% random coil
39	Unidentified	-	-	-	-
	-ARHHGNEFTPVL-	12	33	0, +2	100% random coil
	-SAADKSNVKAAWGKVGGNAGAYGAEAL-	27	48	+1, +1	18.5% alpha helix, 70% random coil
	-FEHFGDLSNADAVMNNPKVKAHGKKVLDS-	29	38	0, +2	17% alpha helix, 83% random coil
42	-VLSAADKSNVKAAWGKVGGNAGAYGAEAL-	29	52	+1, +1	21% alpha helix, 72% random coil
	-FSNGVQHLDDLKGTF-	15	33	-1, 0	13% alpha helix, 60% random coil
	-FEHFGDL-	7	43	-2, -1	100% random coil
46	-WGKVKVDEVGAEALGRL-	17	47	0, 0	47% alpha helix, 35% random coil
48	Unidentified	-	-	-	-
	-LSFPTTKTYFPHF-	13	38	+1, +2	92% random coil
	-TKAVGHLDDLPGTLSDLSDL-	20	35	-3, -2	10% alpha helical, 85% random coil
	-DDLPGTLSDLSDLHAHKLRVDPVNF-	25	36	-3, -1	36% alpha helix, 64% random coil

Fraction Number	Sequence	Number of Amino Acids	% Hydrophobic Residues	Charge at pH 7, 5.5	Secondary Structure Prediction
synthetic	-LAHRYH-	6	50	+1, +3	100% random coil
synthetic	-LAHKYH-	6	50	+1, +3	100% random coil
synthetic	-FQKVVAGVANALAHKYH-	17	59	+2, +4	76.5% alpha helix, 18% random coil
synthetic	-PVNFKLLSHSLL-	12	50	+1, +2	67% random coil

'-' indicates that the property could not be determined due to lack of peptide identity.
% hydrophobic residues and net charge were determined by Peptide Property calculator (www.biosyn.com/PeptidePropertyCalculator/PeptidePropertyCalculator.aspx). Secondary structure predictions were calculated using a consensus secondary structure method of many commonly used algorithms (http://npsa-pbil.ibcp.fr).

Table 6.8 shows that with the exception of the peptide -WGKVKVDEVGAEALGRL- and synthetic -FQKVVAGVANALAHKYH-; all other peptides had extensive random coiled structures i.e. they lack secondary structure. Peptides that were less than 12 amino acids long were 100% random coils. However, the MIC results from this research suggest that simply having random coil structure alone is not sufficient to exert antimicrobial activity. Aside from secondary structure, hydrophobicity and net charge are among the important peptide characteristics affecting antimicrobial activity. For a peptide to exhibit activity it is necessary to possess a certain combination of these factors for interaction with microbial membranes.

There is a range of percentage hydrophobic residues among the peptides from 22 to 59 %, and there appears to be no correlation between the extent of secondary structure and these values. It is widely documented that peptides with a moderate hydrophobicity (around 50% hydrophobic residues) generally exert greater antimicrobial activities. This is due to their ability to permeate the hydrophobic core of the membrane lipid bilayer, which leads to cell death or growth inhibition. Some of the peptides analysed here have this desired moderate hydrophobicity. Above this value, antimicrobial activity is inhibited and toxicity to mammalian cells increases (Yeaman & Yount, 2003).

Converse to the lack of relationship between secondary structure and hydrophobicity found here, Nedjar-Arroume et al. (2008) stated that pepsin generated bovine hemoglobin

peptides with random coil structures have a lower % hydrophobic residues than those that are largely alpha helical. Intuitively it could be thought that this would mean membrane insertion would be more difficult for random coiled peptides and they would therefore exert a lower antimicrobial activity. However, according to Nedjar-Arroume et al. (2008), random coiled peptides exert extremely potent antimicrobial activity, more so than the classical alpha helical AMP class. Random coiled peptides must therefore employ a different antimicrobial mechanism, if they are in fact active. This potent activity was not confirmed by the research in this thesis.

Looking at the peptide charges in Table 6.8, all values are equal or greater under the original test conditions of pH 5.5 in comparison to physiological pH. This is due to the pKa (6.0) of histadine's side group; at pH 5.5 the side chain will be in its protonated form, and hence each histadine residue will contribute an extra positive charge to the peptide. It can also be noted that some of the peptides have zero or negative net charge. This may be responsible for lack of antimicrobial activity in those cases. In terms of antimicrobial activity, a positive peptide charge is important for the initial electrostatic interaction with anionic lipids on the cell wall surface. Therefore, peptides with high positive charges (to an extent) are often associated with high antimicrobial activity. However, when peptides have a very large positive charge, antimicrobial activity becomes diminished and the peptide has greater hemolysis properties (Yeaman & Yount, 2003). From information on amino acid pKa's and knowing that antimicrobial activity is related to charge, it makes sense that the MIC of the peptides tested did not reduce when changing the assay conditions from pH 5.5 to 4.5; the charges on peptides at these two pH are identical.

The one peptide from the ovine hemoglobin digestion that is typical of a classical AMP belongs to fraction 46. It is the only peptide generated that is significantly alpha helical in secondary structure and has a moderate percentage of hydrophobic residues. However, this peptide has zero net charge, which is likely responsible for its low activity. Synthetic -FQKVVAGVANALAHKYH- (beta(129-145)) fits the criteria discussed here for antimicrobial activity of a classical antimicrobial peptide, but for some reason its activity was much lower than expected. As mentioned earlier, a form of modification may be necessary for activity of this peptide.

From further research on the properties of short antimicrobial peptides it appears that the balance between cationic charge and lipophilic bulk is particularly critical for antimicrobial activity. Such peptides require high numbers of arginine and lysine residues (to provide net cationic charge) and tryptophan residues, which provide lipophilic bulk. It seems difficult to obtain a peptide with high quantities of these amino acids by hemoglobin digestion. In such studies, these peptides are created synthetically with amino acid repeats. Furthermore, the synthesised peptides are amidated at the C-terminal ends (Strom et al., 2003 , 2002).

6.2.3.7 Comparison of Peptide Antimicrobial Activity with Similar Peptides from Literature

Some of the peptides generated or synthesised in this research are the same or similar to those reported by other research groups. These were compared in terms of activity against a universally common test organism, *E.coli*, as shown in Table 6.9.

Table 6.9 – Antimicrobial activities of identical or similar hemoglobin peptides from literature, against *E.coli*.

Sequence	Species	Origin	MIC against *E.coli*	Author
-ARHHGNEFTPVL- (Fraction 39)	ovine	114-125 beta-B chain	20-25mg/ml	This research
-ARNFGKEFTPV-	bovine	114-124 beta chain	12uM	Adje et al. (2011)
-SAADKSNVKAAWGKVGGNAGAYGAEAL- (Fraction 39)	ovine	4-30 alpha chain	20-25mg/ml	This research
-VLSAADKSNVKAAWGKVGGNAGAYGAEAL- (Fraction 42)	ovine	2-30 alpha chain	15-18mg/ml	This research
-VLSAADKGNVKAAWGKVGGHAAEYGAEAL-	bovine	2-30 alpha chain	54uM	Nedjar-Arroume et al. (2008)
-VLSPADKTNVKAAWGKVGAHAGEYGAEALERM-	human	2-33 alpha chain	>100ug/ml	Parish et al. (2001)
-VLSPADKTNVKAAWGKVGAHAGEYGAEALERM-	human	2-33 alpha chain	8.2uM	Mak et al. (2000)
-VLSPADKTNVKAAWGKVGAHAGEYGAEALER-	human	2-32 alpha chain	37.0uM	Mak et al. (2000)
-WGKVKVDEVGAEALGRL- (Fraction 46)	ovine	14-30 beta chain	19-24mg/ml	This research
-WGKVKVDEVGGEALGRL-	bovine	14-30 beta chain	Not active	Nedjar-Arroume et al. (2008)
-LSFPTTKTYFPHF- (Fraction 48)	ovine	35-47 alpha chain	12-14mg/ml	This research
-LSFPTTKTYFPHF-	bovine	35-47 alpha chain	9uM	Nedjar-Arroume et al. (2008)
-TKAVGHLDDLPGTLSDLSDL- (Fraction 48)	ovine	68-87 alpha chain	12-14mg/ml	This research
-TKAVEHLDDLPGALSELSDL-	bovine	68-87 alpha chain	Not active	Adje et al. (2011)
-LAHRYH- (synthetic)	ovine	140-145 beta-A chain	Not active	This research
-LAHRYH-	bovine	140-145 beta chain	2uM 45uM	Catiau et al. (2011a), Nedjar-Arroume et al. (2008)
-FQKVVAGVANALAHKYH- (synthetic)	ovine	129-145 beta-B chain	2mg/ml	This research
-QADFQKVVAGVANALAHRYH-	bovine	126-145 beta chain	71uM	Catiau et al. (2011a), Nedjar-Arroume et al. (2008 & 2006)
-PVNFKLLSHSLL- (synthetic)	ovine	96-107 alpha chain	Not active	This research
-VNFKLLSHSLL-	bovine	97-107 alpha chain	MIC: 98uM	Adje et al. (2011)

Note: MIC values for the ovine hemoglobin digestion in this research are given for a fraction not an individual peptide. The highlighted peptide regions are common between peptides from this research and those found in literature.

This information gives further insight into what peptides in the digestion fractions contribute to activity, albeit low. For example, in fraction 42 there are three major peptides to consider (as seen in Figure 6.4); for the most abundant one, alpha(2-30), publications have reported active peptides from this region from bovine and human hemoglobin digestions. Because of the highly conserved nature of the hemoglobin sequence between species, fragments from the same regions of different species are likely to have similar antimicrobial activities.

The data in Table 6.9 suggest that the most abundant peptide in fraction 42 should be active within tens of micromolar concentration. Comparable sequences for the second and third most abundant peptides in this fraction could not be found in literature. However, by looking at their charge characteristics given in Table 6.8, they have zero and a negative net charge under the test conditions respectively. Although anionic AMPs do exist, they require zinc cofactors for activity (Brogden, 2005) and neutral peptides usually have low activity. For this reason it is most likely that peptide alpha(2-30) is responsible for the activity seen from this fraction. However, a much more potent MIC is expected.

Taking into account the reported MIC values of peptides from this region and the charge characteristics of the other peptides in fraction 42, it seems possible that the negatively charged peptides may act antagonistically to alpha(2-30), masking its true activity. Hence, this may be the reason why low activity from this fraction was observed.

The same is likely for fraction 48, which contains negatively charged peptides (as seen in Table 6.8), but also what has been reported to be a very active peptide, alpha(35-47), with an MIC of 9uM against *E.coli*. The region alpha 35 to 47 is 100% conserved between bovine and ovine, and hence this peptide in fraction 48 should have a much greater potency, like the peptide reported in literature. This however, makes the assumption that literature values are accurate.

Moreover, the MIC values for the ovine hemoglobin digest are representative of an entire fraction, not a single peptide, which will greatly underestimate the MIC of an active peptide when inactive fractions are also present. Other reasons accounting for the differences in peptide activity from different groups include microorganism strain, conditions of antimicrobial assays, result interpretation, and the measurement of peptide concentrations in MIC assays.

To calculate an accurate MIC for each peptide species, the hydrolysate needs to be highly separated so that only one peptide species is present in each fraction. If the RP-HPLC gradient allowed for further separation of fractions it is possible that some of the inactive peptides would have been excluded from selection, enhancing MIC values. However, judging from the RP-HPLC profiles, a significant and unpractical lengthening the run time would be needed to further separate fractions. However, if time permitted, the RP-HPLC fractions could be collected and further separated into pure peptides by subsequent RP-HPLC runs with gradients specific to the individual fractions of interest.

In hindsight, it would be recommended to carry out cationic exchange as a preparation step for RP-HPLC to eliminate anionic and neutral peptides first; this would simplify the complexity of the hydrolysate and RP-HPLC fractions, and eliminate peptides that likely have low or no antimicrobial activity.

6.3 Conclusions

The purpose of the work presented here was to determine the antimicrobial activity of native ovine hemoglobin, its apoglobins and short random coiled peptides against a range of common laboratory microorganisms.

Neither native ovine hemoglobin separated from whole blood nor commercial bovine hemoglobin had activity against *E.coli* or *S.aureus* at the highest test concentration of 30mg/ml. This result is in agreement with 'not active' findings by Fogaca et al. (1999) and Mak et al. (2000), despite using the exact protocol as Parish et al. (2001), who reported potent activity of four species of native hemoglobin. The source of extreme variation in the MIC values of native hemoglobin between research groups remains unclear.

In agreement with literature, the separation of ovine hemoglobin subunits and the removal of heme corresponds to a dramatic increase in activity of apoglobins in comparison to the native hemoglobin tetramer. Both ovine apoglobins had moderate activity against *E.coli* and *S.aureus*, but none against *C.albicans* at the highest test concentration of 4mg/ml.

It was found that the majority of RP-HPLC fractions from the urea treated hemoglobin digest had greater activity against the test organism, *E.coli*, in comparison to those from the native hemoglobin digestion. The observation of larger inhibition zones from the urea treated hemoglobin digest fractions may be due to a higher peptide concentration in these fractions, and/or the sequences generated by these kinetics lead to peptides that have characteristics that are more antimicrobially potent.

Fractions 20, 38, 39, 42, 46 and 48 were selected for mass spectroscopy analysis based on their potency against three test organisms and the reproducibility of their peak characteristics. The analysis revealed that each fraction was composed of short peptides ranging from 7-29 amino acids (864-3168Da). However, the fractions contained more than one peptide sequence, and two of the prominent spectra in fractions 39 and 48 were unidentified. It was concluded that this lack of identity is likely due to a chemical modification during sample preparation or processing.

Unlike the results by Nedjar-Arroume et al. (2008), where highly potent small random coiled peptides from bovine hemoglobin pepsin digestion are reported, a lack of potency was demonstrated in the research presented here. The MICs against *E.coli*, *S.aureus* and *C.albicans* were found to be in the milligrams per millilitre range (12-44mg/ml) rather than in ones and tens of micrograms per millilitre. Changes in underlay pH and dilution reagents offered no enhancement of activity.

Synthetic peptides that correspond to sequences from ovine hemoglobin also proved to be limited in their activities, with only beta 129-145 (FQKVVAGVANALAHKYH) showing activity against *E.coli* at the highest test concentration, 2mg/ml. Beta 140-145 (LAHRYH), a random coiled peptide reported by Nedjar-Arroume et al. (2008) to have highly potent activity (2-10uM) against a range of organisms, exerted no activity at the highest test concentration in this research. It is possible that a modification is essential for the activity of these peptides, such as C-terminal amidation.

Analysis of the structural characteristics of the peptides within each fraction confirmed a lack of secondary structure, i.e. random coil peptides, with various net charges and hydrophobicities. The relationship between peptide characteristics and antimicrobial activity appears to be complex. However, further research suggested that a high cationic charge coupled with high lipophilic bulk are essential for antimicrobial activity of short peptides.

A few of the ovine hemoglobin peptides generated here were identical or similar in sequence to those reported in literature, some of which are said to be highly antimicrobial towards *E.coli*. It is hypothesized that inactive peptides within the same fraction as a highly antimicrobial peptide may be antagonistic to that peptides activity. Also, it is likely that the MICs are greatly over estimated as only a portion of the total peptide concentration of each fraction is antimicrobial.

Chapter 7

Conclusions and Recommendations

7.1 Summary of Research Conclusions

This research was carried out to determine the antimicrobial potency of ovine hemoglobin, its subunits and its peptides, with the premise of their use in future antimicrobial products. The objectives of this research were met.

The first objective was to isolate ovine hemoglobin from fresh whole blood in order to obtain apoglobins and short random coiled peptides for antimicrobial testing. Literature has suggested that heme-free globins have greater antimicrobial activity than native hemoglobin itself (which may or may not possess activity), and that short hemoglobin peptides can be extremely potent towards certain microorganisms.

Erythrocytes were lysed within the whole blood solution using isotonic ammonium chloride, followed by centrifugation. Although this protocol allowed for fast and simple separation of ovine hemoglobin from whole blood, by observation a lot of hemoglobin was lost upon decanting the plasma layer. This method was acceptable for such a small scale experiment, especially due to the high concentration of hemoglobin per erythrocyte. However, it is recommended that another isolation protocol should be used in the case of up-scaling to maximise the hemoglobin yield obtained and minimise wastage. Furthermore, the mass spectrometry presented in chapter six showed that the plasma protein, serum albumin, was present in the digestion reactions (data for this is not shown). When testing the antimicrobial potency of the intact hemoglobin this could potentially cause some inhibition of activity. Moreover, due to the similarity in size between ovine hemoglobin (66kDa) and ovine serum albumin (69kDa), it would be difficult to separate the two proteins by a simple method such as gel filtration. It is concluded that the isolation of the erythrocytes from the whole blood first would enable erythrocytes to be washed free of any plasma or WBC

contaminants before the lysis takes place. This would also result in greater hemoglobin yields.

From the isolated hemoglobin, apoglobins were obtained by a common method, acid acetone precipitation, as the conditions of RP-HPLC alone failed to dissociate ovine hemoglobin subunits. The remainder of the hemoglobin solution was digested into peptides by pepsin at pH 4.5, which is a compromise between pepsin activity and retaining native hemoglobin conformation. A second substrate conformation was achieved by the addition of urea, which acts to denature hemoglobin. Tricine SDS-PAGE gels showed the pepsin digestion of the two substrates over time; in the presence of urea, ovine hemoglobin was digested much more rapidly and with different digestion kinetics in comparison to the native hemoglobin substrate.

The second objective was to purify the apoglobins and hemoglobin pepsin digests by RP-HPLC. Apoglobins were separated into alpha and beta fractions by loading the acid acetone precipitate onto a semi-preparative RP-HPLC column. RP-HPLC chromatograms for each time point in the hemoglobin pepsin digest, from both digestion conditions were obtained using an analytical column. The profiles reflected the findings of the tricine SDS-PAGE gels mentioned above. Native hemoglobin was digested according to the one by one mechanism suggested in literature, whilst the urea treated hemoglobin was expected to be digested following the zipper mechanism. However, although the urea treated hemoglobin was digested at a considerably faster rate than the native hemoglobin, it was not nearly as fast as suggested by literature and only a few intermediates were identified. This combined with the absence of two protein fractions in the RP-HPLC chromatograms (reflecting the lack of alpha and beta subunit dissociation) suggests that ovine hemoglobin was not fully denatured by 5.3M urea. The little information available on the stability of sheep hemoglobin suggests that the tetramer is more stable than the hemoglobin tetramer from most species. To achieve ovine hemoglobin dissociation and denaturation, it is recommended to further increase the urea concentration or denature the hemoglobin under strongly acidic conditions.

Semi-preparative RP-HPLC was carried out to obtain adequate peptide product for antimicrobial testing. However, due to time and cost restrictions only one time point was

analysed. The 24hr digestion was selected as it contained the highest peptide yield, and most likely a larger concentration of small random coiled peptides due to the greater extent of digestion.

The next objective was to determine the antimicrobial activities of ovine hemoglobin, its apoglobins, and its peptides from pepsin digestion. It was found that the native ovine hemoglobin obtained from this research and powdered bovine hemoglobin exerted no antimicrobial activity against *E.coli* or *S.aureus*. They exerted no activity at the highest test concentration of 30mg/ml in the radial diffusion assay system. This result was in agreement with Mak et al. (2000), who found human hemoglobin and other heme-containing proteins to be antimicrobially inactive against a range of test organisms.

The ovine apoglobins prepared from the acid acetone precipitation of ovine hemoglobin were moderately active against *E.coli* and *S.aureus*, with both purified apoglobin fractions exerting MICs of 0.5-1.0mg/ml. However, neither fraction was active towards the fungi, *C.albicans* at the highest test concentration of 4.0mg/ml. In agreement with literature findings, ovine hemoglobin apoglobins were many times more antimicrobially active than the native tetramer. It is likely that the dissociation of the tetramer into monomers, and perhaps to a lesser extent, the removal of heme, results in the exposure of new antimicrobial surfaces which were previously hidden.

The RP-HPLC fractions of the 24hr hemoglobin digestion in the presence of urea proved to be more inhibitory towards *E.coli* than fractions from the non-urea digest, as shown by the significantly larger inhibitory zones. Therefore, the activities of the urea digest fractions were further analysed against two other test organisms, *S.aureus* and *C.albicans*, to aid in the selection of the most promising candidates for mass spectrometry and further testing. The majority of fractions were active towards *E.coli* and *S.aureus*, but only one was active against *C.albicans*. Fractions 20, 38, 39, 42, 46, and 48 were chosen for mass spectrometry based on their significant activities against more than one test organism, and their resolution on the chromatograms, which ensures consistency in composition between RP-HPLC runs. Mass spectrometry identified these peptides as short, between 7-29 amino acids in length. Obtaining small peptides was one objective of this research. However, two of the most abundant peptides in fractions 39 and 48 were unidentified by the database search. It was

concluded that this could be due to one of the hundreds of artificial or natural modifications. All six fractions showed weak antimicrobial activity, being most effective towards *E.coli*, followed by *S.aureus*, with only fraction 38 active towards *C.albicans*. The lowest MIC value was high at 12mg/ml.

Aside from one synthetic ovine hemoglobin peptide, beta-B(129-145), no others displayed activity in the radial diffusion assay. Furthermore, neither the synthetic nor the digested peptides exerted any activity in the microtitre broth assay. In the case of the synthetic peptides, some of which were shown in literature to be highly active, perhaps a modification is essential for their activity.

From the mass spectrometry data, peptide secondary structure, charge, and the percentage of hydrophobic residues were determined. The majority of the peptides were in fact random coils, however, some were positively charged whilst some had no charge or were negatively charged at the test pH. Furthermore, the hydrophobicities of the random coiled peptides were not as low as suggested by Nedjar-Arroume et al. (2008). When comparing the activities of the peptides generated in this research with similar hemoglobin peptides found in the literature, a few of the abundant peptides were said to be highly antimicrobial, against *E.coli* at least. However, this was not observed in the research presented here. It is thought that the antimicrobial activity of any active peptide may be masked by other peptides within that fraction i.e. negatively charged peptides. Moreover, the MIC values for the peptide digest are representative of an entire fraction, not a single peptide, which will greatly underestimate the MIC of an active peptide. It is recommended that cation exchange chromatography be carried out as a preparative step for RP-HPLC. This would eliminate peptides with negative or no charge, which are likely to be inactive and cause great complexity of the RP-HPLC fractions. As a result, MIC values would be more representative of any active peptides within a fraction.

It is concluded that native ovine hemoglobin is not antimicrobial *in vitro*, but the separation of its subunits exposes hidden antimicrobial surfaces, leading to significant microbial killing or growth inhibition. Furthermore, the results of this research showed that short random coiled peptides from pepsin hydrolysis of ovine hemoglobin are only weakly antimicrobial. This is in contrast to evidence presented by Nedjar-Arroume et al. (2008) where small

random coiled peptides from bovine hemoglobin digestion are extremely potent, more so than classic alpha helical peptides. The lack of peptide activity presented here is supported by Mak et al. (2000) who found that chemical or enzymatic fragmentation of hemoglobin into peptides smaller than 50 residues results in reduced or abolished antimicrobial activity.

7.2 Recommendations for Future Research

This research contributes to, and enhances the limited knowledge on the antimicrobial potency of ovine hemoglobin, its apoglobin and its peptides. However, the peptide size and structural characteristics that confer to potent antimicrobial activity remain unclear, as do many properties of AMPs. The results of the work presented here give insight into new approaches that could be tried in future research; such research includes:

1. Implementation of a hemoglobin recovery method from whole blood that results in high hemoglobin yield and lacks plasma protein and/or WBC contamination.

2. Identify conditions that will denature ovine hemoglobin without affecting pepsin activity; analyse the effects of increasing urea concentration or using strongly acidic conditions on ovine hemoglobin conformation. This research showed that the digestion of denatured hemoglobin rapidly increases the reaction rate, peptide yield, and perhaps results in peptides with greater antimicrobial activity.

3. Carry out cationic exchange as a preparative step for RP-HPLC. This will eliminate peptides with net zero or negative charge, which are likely to be low in activity. This will simplify the complexity of the hydrolysate and make purification of individual peptides easier.

4. Shift focus to the generation and analysis of all peptide secondary structures, not just small random coiled peptides, as these were shown to be only weakly antimicrobial. Larger peptides may be more active, as suggested by Mak et al. (2000). Therefore, collecting RP-HPLC fractions from earlier degrees of hydrolysis is also recommended.

5. Carry out MIC testing against a wider range of test organisms. Peptide activity may be directed at specific species of microorganisms that were not tested here.

6. Determine the effect of common factors antagonistic to peptide activity, such as, monovalent and divalent cation concentration, salt concentration, pH and temperature. These are factors which can diminish the antimicrobial activity of a peptide *in vivo* and after product processing.

7. Investigate whether synergy occurs between different ovine hemoglobin AMPs or between ovine hemoglobin AMPs and existing antibiotics. The presence of synergy would mean less total peptide would be required to achieve the same level of antimicrobial activity. Also, if synergy occurs it is likely that the two or more peptides involved have different mechanisms of action and it is therefore much more difficult for microbes to overcome their activity and develop resistance.

8. Determine the mechanism of antimicrobial activity by the peptides. This may be through targeting the cell membrane via carpet-like, barrel-stave, or toroidal-pore mechanisms, or by interaction with intracellular targets. The action may inhibit growth or cause cell death i.e. bacteriostatic or bacteriocidal.

9. Cytotoxcity, hemolysis, LPS binding and immunological properties are essential parameters to determine, as they govern the acceptability of using AMPs *in vivo*. These factors should therefore be investigated.

10. A comparison of antimicrobial activities between the generated peptides and synthetic peptides would also be of interest, allowing for insight into the contribution of modifications to peptide activity.

Assuming highly potent antimicrobial peptides that function *in vivo* can be generated from ovine hemoglobin, they must also be commercially viable. Such factors that need to be considered are:

1. Determining whether protocols for generating ovine hemoglobin peptides can be successfully upscaled.

2. Is the production of these peptides commercially viable?

3. What products would these peptides best suit? As mentioned in the literature review, currently the use of AMPs as therapeutics is limited to topical application due to the unknown toxicology of systemic use. They could add-value in topical products for the treatment of cuts and grazes, burns, eczema, acne, body ulcers, and STDs. However, their application(s) would be dictated by what microorganisms they can inhibit or kill.

4. Forming a working relationship with sheep slaughtering companies, and implementing an appropriate procedure for obtaining sheep blood that is accepted by both parties.

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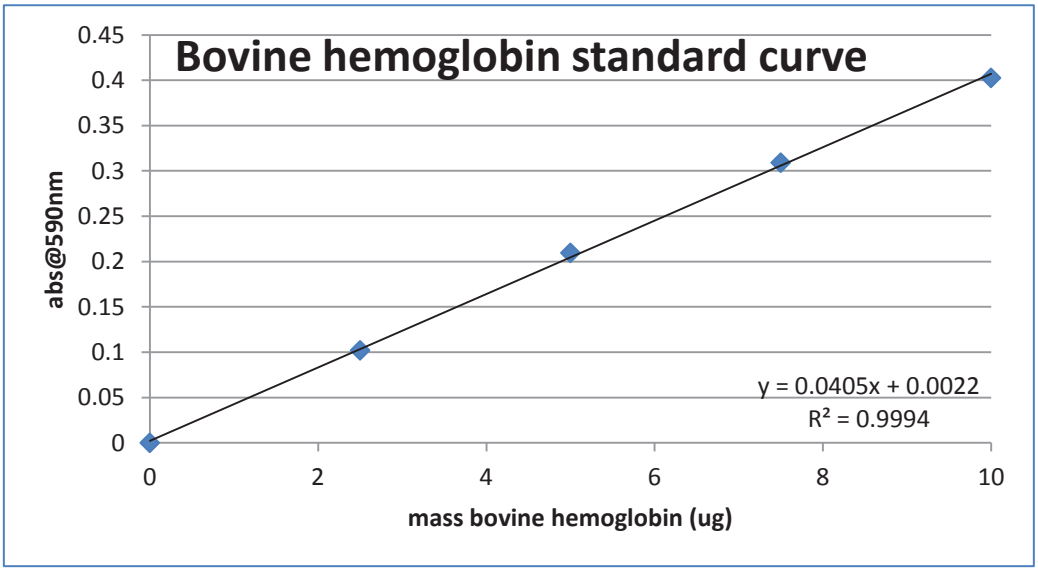
Appendix 1

Raw Data and Calculations from the Generation of Apoglobins and Peptides from Native Ovine Hemoglobin

1.1 Calculation of the Ovine Hemoglobin Concentration after Extraction from Whole Blood

Powdered bovine hemoglobin was available to use as a protein standard in the determination of ovine hemoglobin concentration after the extraction from whole blood.

	Absorbance at 590nm		
Mass of Bovine Hemoglobin (ug)	Absorbance 1	Absorbance 2	Average Absorbance
10	0.400	0.405	0.403
7.5	0.309	0.309	0.309
5	0.208	0.211	0.210
2.5	0.103	0.101	0.102
0	0	0	0



Calculation:

Isolated ovine hemoglobin solution was diluted $5x10^4$ times to fall within the standard curve absorbance range.

Abs @ 590nm = 0.112, 0.108

= 0.11

Abs = 'y' in standard curve equation, 'x' = ug of ovine hemoglobin

Therefore:

0.11 = 0.0405x + 0.002

x = 2.66ug ovine Hb in 500ul of diluted sample

x = 133086ug ovine Hb in 500ul undiluted sample (DF = 50000)

x = 266173ug ovine Hb/ml

= 266mg/ml

1.2 Determination of Ovine Hemoglobin Quantity required for Pepsin Digestion

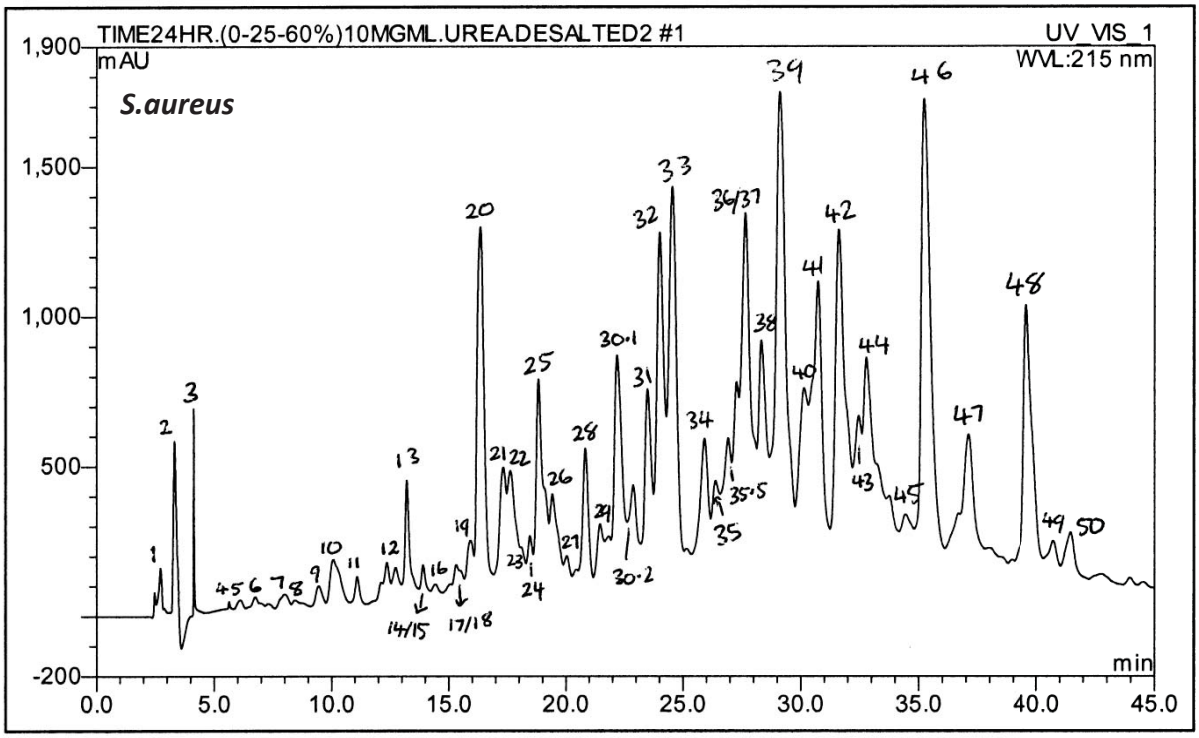
Following a method by Nedjar-Arroume et al. (2008) and Su et al. (2007b), the hemoglobin solution was to be digested at a concentration of 10mg/ml. Therefore, the solution was diluted 2.7 times with sodium acetate buffer before digesting with 1mg/ml porcine pepsin.

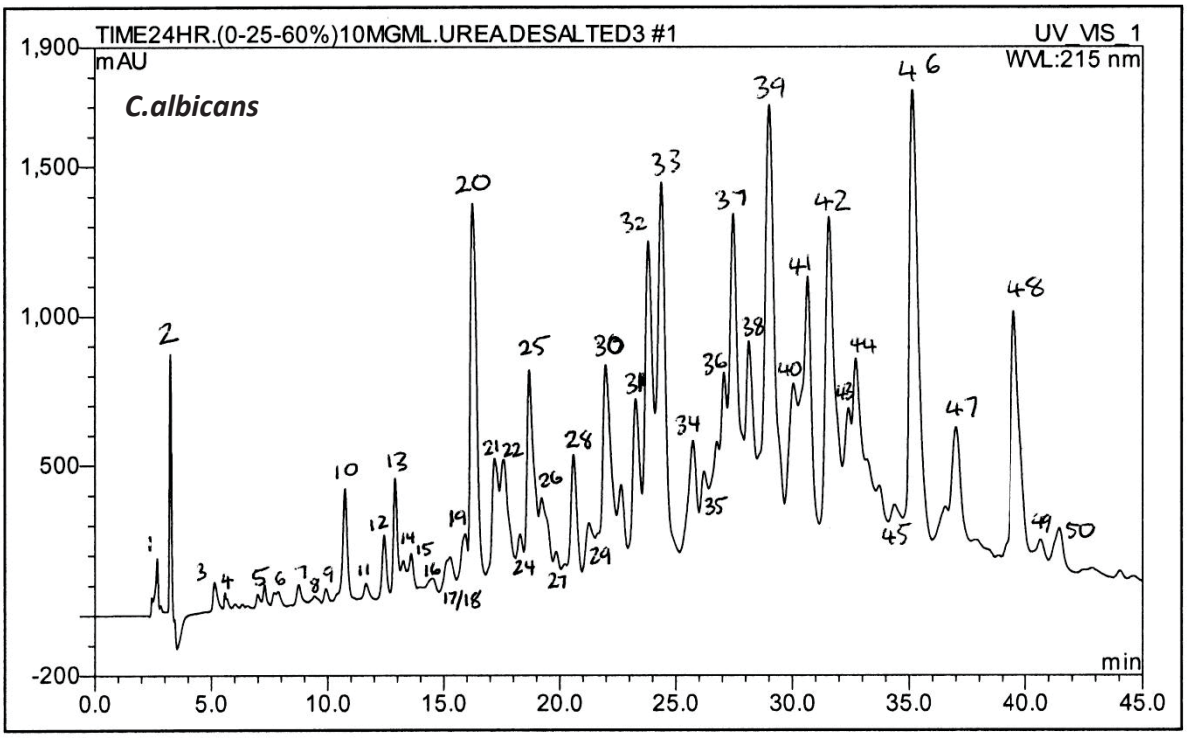
Appendix 2

Raw Data and Calculations from the Purification and Antimicrobial analysis of Ovine Hemoglobin, its Apoglobins and Peptides

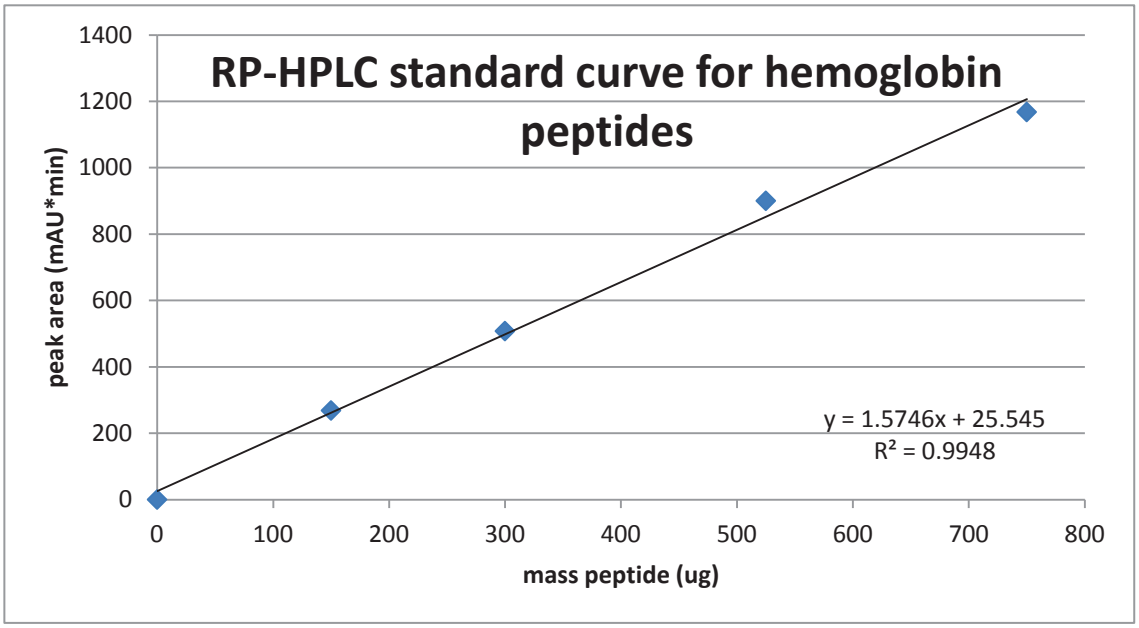
2.1 Semi-preparative RP-HPLC Profiles of 24hr Urea Treated Ovine Hemoglobin Pepsin Digests

These fractions were collected and assayed against *S.aureus* and *C.albicans*, respectively, to obtain inhibition zone readings shown in Table 6.5.





2.2 Example Calculations for the Determination of Peptide Yields of RP-HPLC Fractions



From the RP-HPLC standard curve:

Peak area = 1.5746*(Peptide mass) + 25.545

Therefore:

Peptide mass = (Peak area – 25.545)/1.5746

Example – a collected fraction has a peak area of 490.7mAU*min:

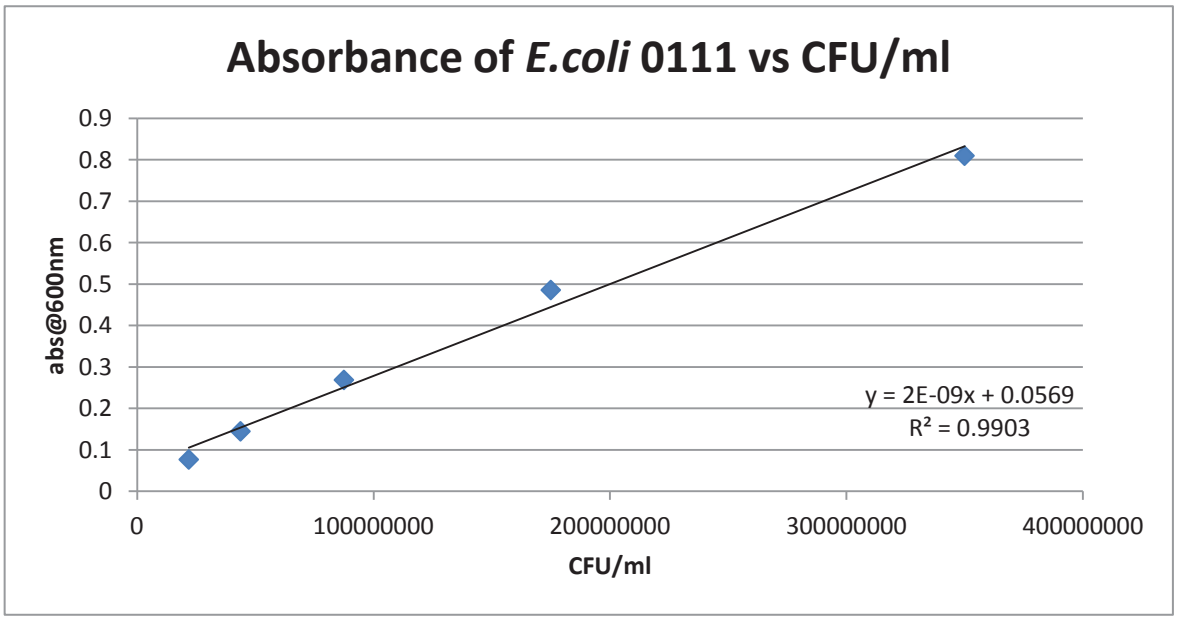
Peptide mass = (490.7 – 25.545)/1.5746 = 295.4 ug of peptide

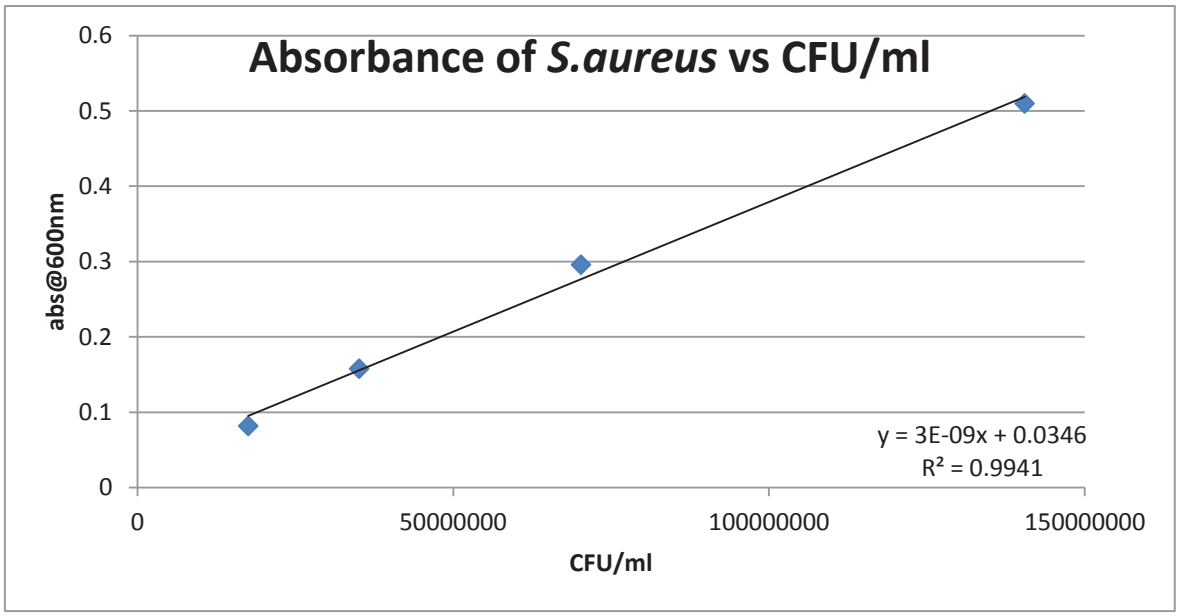
Note: Peak area is provided by the RP-HPLC output

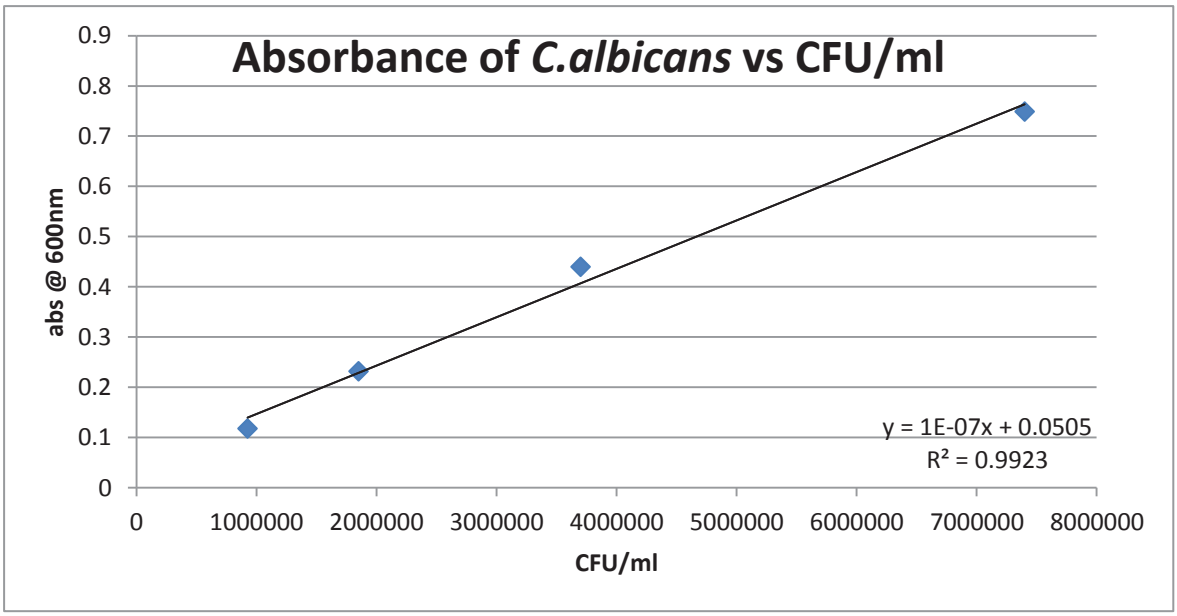
Concentration when reconstituted in 15ul of 0.01% acetic acid:

Concentration = 295.4ug x 1000/15 = 19692ug/ml = 19.7mg/ml

2.3 Logarithmic Growth Profiles of Test Organisms




Absorbance of S.aureus vs CFU/ml
abs@600nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50000000
100000000
150000000
CFU/ml
y = 3E-09x + 0.0346
R² = 0.9941


Absorbance of C.albicans vs CFU/ml
abs @ 600nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1000000
2000000
3000000
4000000
5000000
6000000
7000000
8000000
CFU/ml
y = 1E-07x + 0.0505
R² = 0.9923